

IMMUNOMODULATION
BY ADIPOKINES
IN TYPE 1 DIABETES

By

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Abstract

Type 1 diabetes (T1D) is a T cell-mediated autoimmune disease in which the immune system specifically targets and destroys the pancreatic insulin-producing beta-cells. We are interested in defining whether adipose tissue-derived cytokines (adipokines) such as adiponectin (anti-inflammatory) and leptin (pro-inflammatory) could influence T1D progression.

We first demonstrate the expression of the leptin receptor (LEPR) on peripheral blood mononuclear cells (PBMC) and observed higher expression of LEPR on PBMC from patients with T1D. However, we found no significant functional relevance for this observation. On the other hand, we show lower expression of the adiponectin receptors on lymphocytes from patients with T1D. This was associated with a reduced capacity of adiponectin to inhibit lymphocyte trans-endothelial migration in T1D. Remarkably, we show that adiponectin strongly inhibited lymphocyte migration either by action on the endothelium or directly on the lymphocytes. We have now established that this effect on lymphocytes is mediated by a newly identified B cell-secreted agent following adiponectin stimulation. The agent stimulates endothelial production of sphingosine-1-phosphate, which in turn is responsible for the inhibition of lymphocyte trans-endothelial migration. These observations were confirmed *in vivo* using a peritoneal model of inflammation in a B cell knock-out mouse strain. In these animals, T cell recruitment in the peritoneum is increased compared to the wild-type and restored when the agent was administrated.

These observations underline the importance of adiponectin in the control of lymphocyte transmigration during an inflammatory response and offer a potential therapeutic agent for T1D as well as other T cell-mediated diseases.

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Abbreviations

Acrp30: Adipocyte complement-related protein of 30 kDa

ADA: American Diabetes Association

AMPK: 5' adenosine monophosphate-activated protein kinase

ANOVA: Analysis of variance

APC: Allophycocyanin

APC-Cy7: Allophycocyanin Cyanine 7

APCs: Antigen presenting cells

ApoE: Apolipoprotein E

APPL1: DCC-interacting protein 13-alpha

AQ: Adiponectin

AR1/2: Adiponectin receptors 1 and 2

ATP: Adenosine triphosphate

BCR: B cell receptor

BMI: Body mass index

Bp: base pair

BPC: Base peak chromatogram

BSA: Bovine serum albumin

CD: Cluster of differentiation

cDNA: Complementary deoxyribonucleic acid

CFSE: Carboxyfluorescein succinimidyl ester

CID: Collision-induced dissociation

Ct: Threshold cycle

CTLA-4: Cytotoxic T-Lymphocyte Antigen 4

CVD: cardiovascular disease

Da: Dalton

db: leptin receptor gene

DCs: Dendritic cells

DC-SIGN

dNTPs: Deoxyribonucleotide triphosphate

DP-1: Prostaglandin receptor 1

DP-2: Prostaglandin receptor 2

EAE: Experimental autoimmune encephalomyelitis

EC: Endothelial cells

EDTA: Ethylenediaminetetraacetic acid

eNOS: Nitric oxide synthase

ERK-1/2: Extracellular-signal-regulated kinases 1/2

ESI: Electrospray

FITC: Fluorescein isothiocyanate

FoxP3: Forkhead box P3

FTY720: Fingolimod

GAD65: Glutamic acid decarboxylase 65

gDNA: genomic deoxyribonucleic acid

Abbreviations

GDR: Glucose disposal rate
GM-CSF: Granulocyte-macrophage colony-stimulating factor
GPCR: G-protein coupled receptor
HbA1c: Glycated haemoglobin
HC: Healthy control
HDL: High density lipoprotein
HEV: High endothelial
HLA-DQ: human leukocyte antigen DQ
HLA-DR: human leukocyte antigen DR
HMW: High molecular weight
HOMA: Homeostasis Model Assessment
HPRT1: Hypoxanthine phosphoribosyltransferase 1
HTN: Hypertension
HUVEC: Human umbilical vein endothelial cells
IA-2: Islet antigen-2
IAA: InsulinAutoantibody
ICAM-1: Intercellular Adhesion Molecule 1
ICOS-L: Inducible costimulator-ligand
IFN- γ : Interferon-gamma
Ig: Immunoglobulin
IL: Interleukin
INS: Insulin
IR: Insulin resistance
iTreg: induced regulatory T cells
JAK: Janus Kinase
JAM-A, B, C: Junctional adhesion molecule A, B, C
KO: knock-out
LAG-3: Lymphocyte-activation gene 3
LC: Liquid chromatography
LEPR: Leptin receptor
LFA-1: Lymphocyte function-associated antigen 1
/LEPR: Long leptin receptor isoform (human)
LMW: Low molecular weight
LPS: Lipopolysaccharide
m/z: Mass/charge
MAPK: Mitogen-activated protein kinases
MCP-1: Monocyte chemotactic protein-1
MFI: Mean fluorescence intensity
MHC: Major histocompatibility complex
MIP-1 α : Macrophage inflammatory protein-1
MMW: Medium molecular weight
mRNA: Messenger ribonucleic acid

Abbreviations

MS/MS: Tandem mass spectrometry
NA: Not applicable
NF- κ B: nuclear factor kappa-light-chain-enhancer of activated B cells
NHS: National health system
NK: Natural killer
NO: Nitric oxide
NOD: Non obese Diabetic
Nrp-1: Neuropilin-1
nTreg: natural regulatory T cells
ob: leptin gene
ObR: Leptin receptor (mouse)
PB: Pacific blue
PBL: Peripheral blood lymphocytes
PBMC: peripheral blood mononuclear cells
PBS: Phosphate buffered saline
PBSA: Phosphate buffered saline plus albumin
PCR: Polymerase chain reaction
PDL-1: Programmed cell death 1 ligand 1
PE: Phycoerythrin
PECAM1: Platelet endothelial cell adhesion molecule 1
PECy7: Phycoerythrin Cyanine 7
PerCpCy5.5: Peridinin-chlorophyll-protein complex tandem with Cyanine 5.5
PGD2: Prostaglandin D2
PHA: Phytohaemagglutinin
PI: Propidium Iodide
PPAR- α : Peroxisome proliferator-activated receptor alpha
PSGL-1: P-selectin glycoprotein ligand-1
PTPN-22: Protein tyrosine phosphatase, non-receptor type 22
 R^2 : coefficient of determination
RANTES (CCL5): Regulated upon Activation, Normal T-cell Expressed, and Secreted
RBC: red blood cells
RPMI: Roswell Park Memorial Institute
S1P: Sphingosine-1-phosphate
S1PR: Sphingosine-1-phosphate receptor
sLEPR: Short leptin receptor isoform (human)
SLOs: Secondary lymphoid organs
SPHKs: Sphingosine-1-phosphate kinases
STAT: Signal transducer and activator of transcription
T1D: Type 1 diabetes
T2D: Type 2 diabetes
TCR: T cell receptor
TGF- β : Transforming growth factor beta

Abbreviations

Th1: T helper 1

Th2: T helper 2

TLR: Toll-like receptor

TNF- α : Tumour necrosis factor alpha

Treg: regulatory T cells

UK: United Kingdom

VCAM-1: Vascular cell adhesion protein 1

VE-cadherin: Vascular endothelial cadherin

VLA-4: Very Late Antigen-4

WHR: Waist-Hip ratio

wt: wild-type

ZnT8: Zinc transporter 4

1.Chapter 1- GENERAL INTRODUCTION

1. Type 1 diabetes (T1D) disease

Diabetes mellitus is a group of diseases characterised by high circulating blood glucose. T1D is a chronic autoimmune disease in which the immune system specifically targets and destroys the insulin-producing beta-cells, located in the pancreatic islets of Langerhans. This results in loss of insulin secretion (Daneman *et al.*, 2006). Insulin is an important metabolic hormone that regulates glucose uptake, and insulin deficiency resulting from the autoimmune process leads to high blood glucose.

1.1. Clinical aspects of T1D

Studies in relatives of patients at high risk of developing T1D have shown that the beta-cell loss resulting in T1D can last from months to years before the disease is clinically detected (DCCTRG, 1998; Achenbach *et al.*, 2006) (**Figure 1-1**). At clinical diagnosis, 50 to 80% of the beta-cell function has been lost. After initial insulin treatment, a few patients can exhibit an insulin-independent phase (honeymoon) where insulin injections are drastically reduced or even stopped (Abdul-Rasoul *et al.*, 2006). The occurrence of honeymoon relies on factors associated with preservation of beta-cell function, such as the age at diagnosis, number of different auto-antibodies present, body mass index (BMI), genetic risks factors, glycated haemoglobin (HbA1c), C-peptide and immune parameters (level of interleukin-1 (IL-1) receptor, IL-1 beta for example) (reviewed in Ali *et al.*, 2009). When beta-cell function falls below a threshold required for maintaining euglycaemia, patients exhibit symptoms caused by hyperglycaemia such as polyuria, polydipsia, tiredness and weight loss, sometimes with polyphagia and blurred vision (ADA, 2004; Roche *et al.*, 2005).

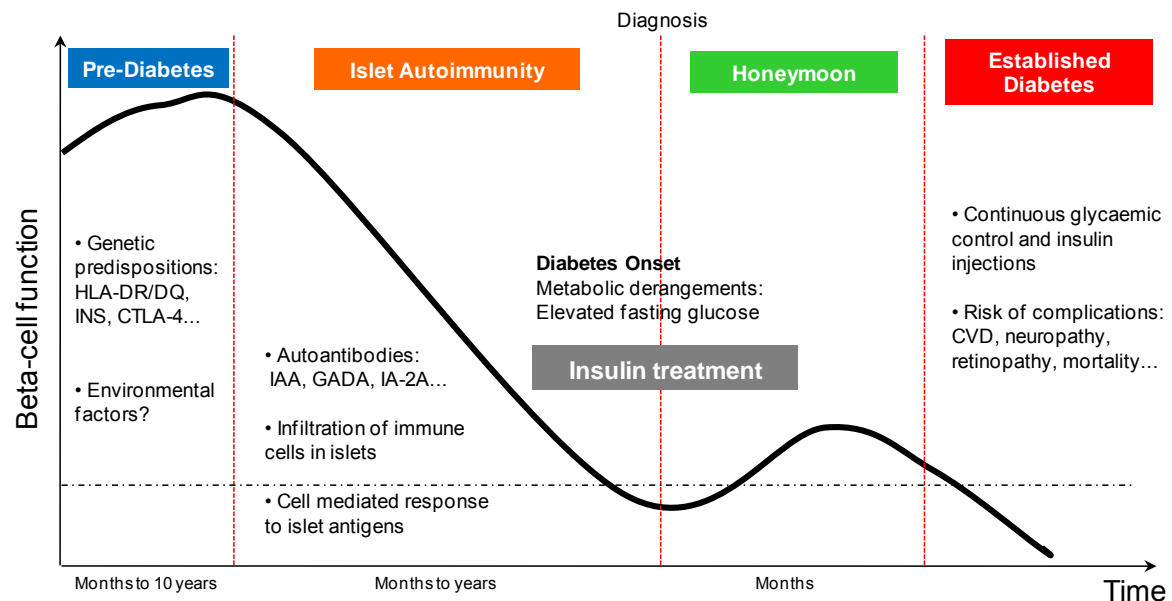


Figure 1-1: Natural history of T1D

T1D has both genetic predispositions and environmental factors. An as yet unidentified trigger elicits islet autoimmunity in individuals at genetic risk for T1D. Islet autoimmunity manifests as the appearance of autoantibodies against islets antigens such as insulin (IAA), glutamic acid decarboxylase (GAD65) and the tyrosine phosphatase-like protein (IA-2). There is a subsequent infiltration of immune cells into the islets. This initiates a gradual loss of beta-cell function that can take years before clinical symptoms appear. After beta-cell function has fallen below a threshold, classic symptoms of diabetes caused by hyperglycaemia are detectable, leading to the diagnosis of T1D. Some patients experience a remission referred as the honeymoon phase after initial insulin treatment. This phase can last months or even a year, but beta-cell function is rapidly degraded as the islet autoimmunity progresses.

If not rapidly treated, these symptoms will quickly result in dangerous acute complications such as ketoacidosis (Neu *et al.*, 2003). Long-term complications include retinopathy leading to blindness; peripheral neuropathy with risk of foot ulcers, amputations and Charcot joints; neuropathy causing gastrointestinal, genitourinary and sexual dysfunction; nephropathy resulting in renal failure; cardiovascular disease and high risk of mortality (ADA, 2004; Soedamah-Muthu *et al.*, 2006). More than 80% of the subjects have residual beta-cell function in the first year following diagnosis (Greenbaum *et al.*, 2009). This falls to 10-20% within five years of disease and to 1% after five years (Scholin *et al.*, 2004), though there appears to be considerable variability in these figures. Residual beta-cell function

improves HbA1c, lowers risk of hypoglycaemia, reduces long-term complications, decrease risks of ketoacidosis and improves the response to immunotherapy (reviewed in Ali *et al.*, 2009). This indicates the importance of preserving beta-cell function, even at low levels, for as long as possible (Torn *et al.*, 2000).

1.2. Prevalence of T1D

Despite a better understanding of the natural history of T1D, its incidence continues to increase in the United Kingdom (UK) and worldwide, especially among children under the age of 5 years (**Figure 1-2**) (Gardner *et al.*, 1997; Moore *et al.*, 2003; Zipris *et al.*, 2009). Northern Europe, North America, New Zealand and Australia exhibit the highest rates, whilst much lower incidence is observed in Asia, Africa and South America (DIAMOND project group, 2006). 2.6 million people have currently been diagnosed with diabetes in the UK, and by 2025 this number is predicted to increase to 4 million (**Figure 1-2c**) (DUK, 2010). These figures include both T1D and type 2 diabetes (T2D). Including both children and adults, it is estimated that 15% have T1D.

Alarmingly, on average 400 people per day are diagnosed with diabetes (including T1D and T2D) in the UK. This impacts the national health system (NHS) budget. The latest report states that about £9 billion per year is spent on diabetes, which represents about 10% of the NHS annual expenditure (NHS, 2011).

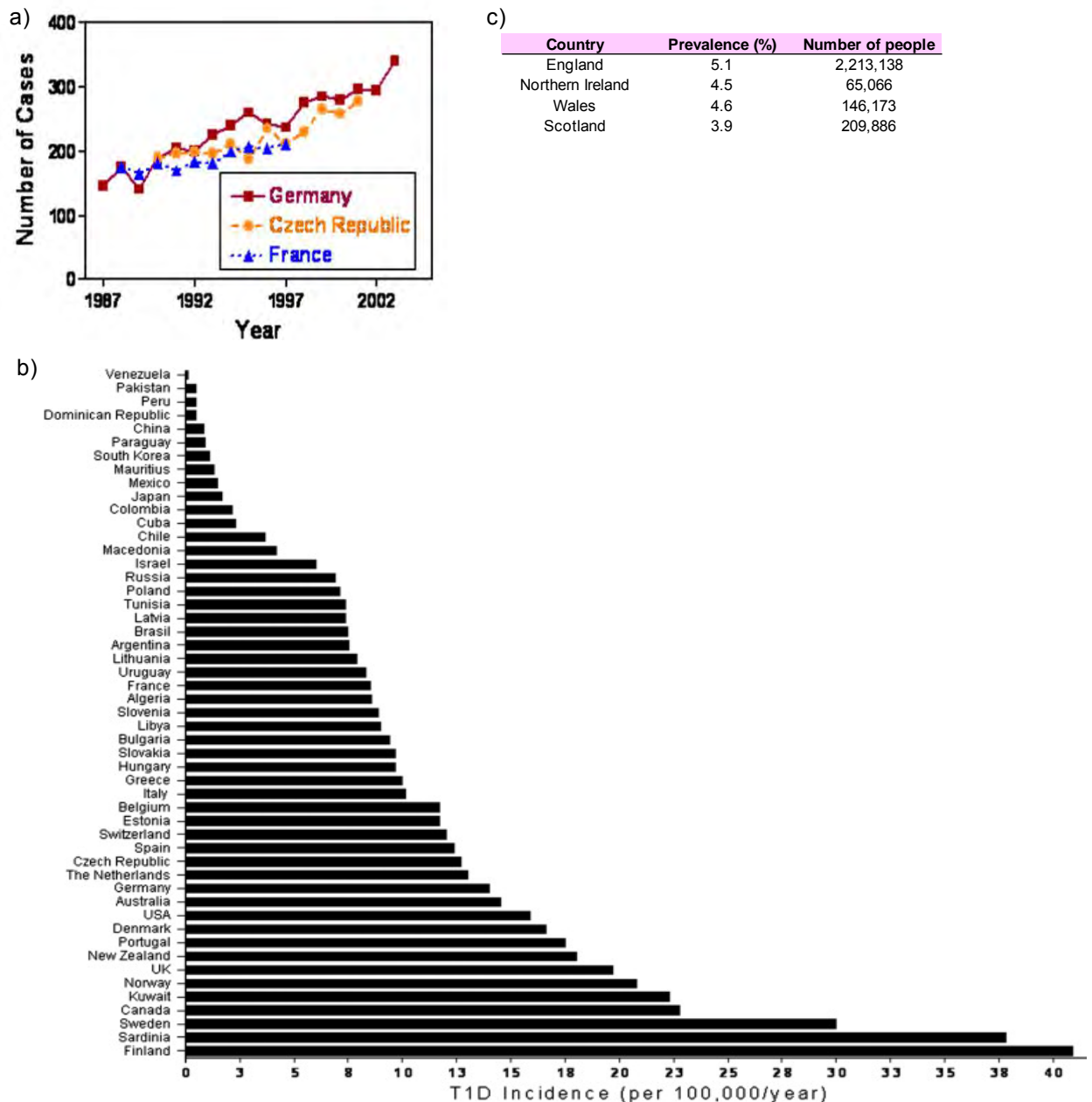


Figure 1-2: Global prevalence of T1D

(a) Numbers of cases of T1D have significantly increased in European countries such as Germany, Czech Republic and France (Zipris *et al.*, 2009; copyright license number: 2814281274788) (b) but also in other develop countries such as Australia, New Zealand and North America (Zipris *et al.*, 2009; copyright license number: 2814281274788). (c) In the UK, the prevalence of diabetes in 2009 was a total of 2.6 million people. However, these statistics include both type 1 and type 2 diabetes. Within the 2.6 million of people with diabetes, it is estimated that 15% have type 1 diabetes (DUK, 2010).

1.3. Immunity and pathogenesis of T1D

1.3.1. Autoimmunity in T1D

T1D is characterised by the auto-immune destruction of insulin-producing pancreatic beta-cells, a process referred to as insulinitis (Cnop *et al.*, 2005).

1.3.1.1. Humoral autoimmunity in T1D

The autoimmune destruction of beta-cells is associated with the appearance of islet auto-antibodies early in life (Kimpimäki *et al.*, 2001).

Auto-antibody production can be detected up to five years before clinical diagnosis of T1D, making them an attractive marker of diabetes risk and a tool for diagnosis (Lindberg *et al.*, 1999). The most common auto-antibodies are directed against the glutamic acid decarboxylase (GAD65), the tyrosine phosphatase-like protein (IA-2) and insulin itself (IAA) (Velloso *et al.*, 1993; Lan *et al.*, 1996; Palmer *et al.*, 1983). Studies have shown that high risk of developing T1D is associated with subjects carrying auto-antibodies to two or three of these antigens (Verge *et al.*, 1998). Combination of GAD65 and IA-2 is linked to a 50% risk, GAD65, IA-2 and IAA to at least 70% of developing diabetes (Leslie *et al.*, 1999; Verge *et al.*, 1996). This risk is increased when combining the detection of these antibodies with detection of the recently discovered beta-cell antigen ZnT8, a zinc transporter (Wenzlau *et al.*, 2007).

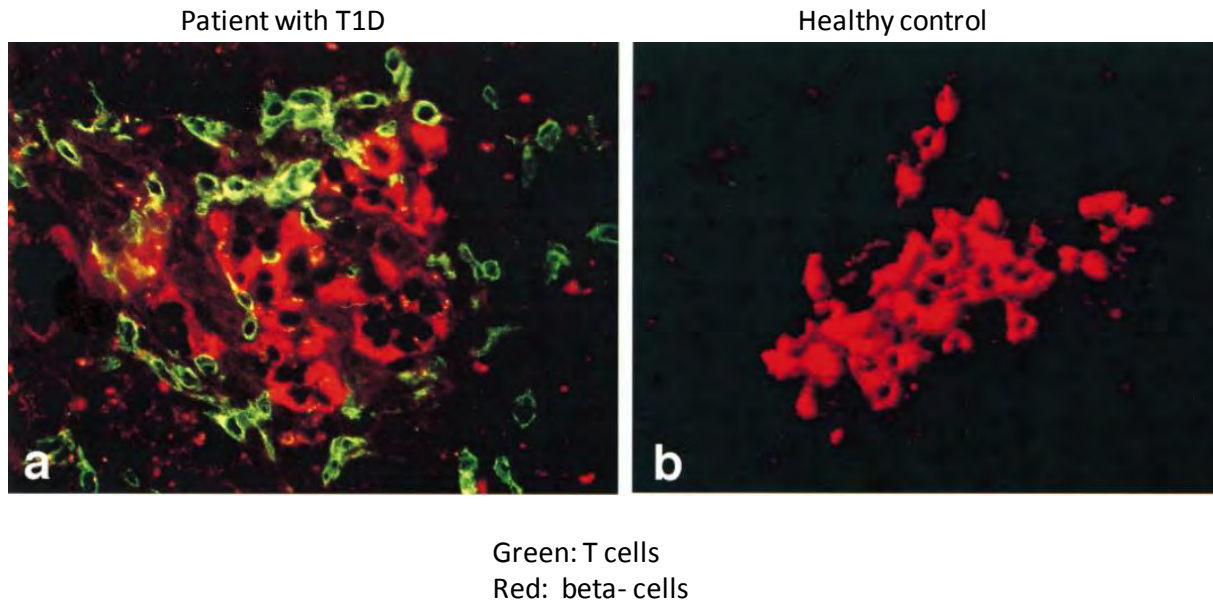


Figure 1-3: Insulitis in the pancreatic islet resulting in auto-immune destruction of beta-cells

Section of the pancreatic islets stained for insulin (red) and CD3 (cluster of differentiation 3), a marker of T cells (green). This shows infiltration of T cells in the pancreas from a patient with T1D (left) compared to a healthy subject without infiltration (right) (Imagawa *et al.*, 1999; copyright license number: 2814290215519).

1.3.1.2. Cellular autoimmunity in T1D

The destruction of beta-cells is predominantly mediated by T cells. This is evidenced by the presence of T cells in the insulitic lesions (**Figure 1-3**) (Imagawa *et al.*, 1999), and by the presence of autoreactive circulating T cells (Hanafusa *et al.*, 2008). Studies in humans and murine models have demonstrated that both $CD4^+$ and $CD8^+$ T cells subsets, but also macrophages, monocytes, dendritic cells (DCs) and B lymphocytes are required to trigger insulitis (Xiu *et al.*, 2008; Lee *et al.*, 1998; Papaccio *et al.*, 1999; Miyazaki *et al.*, 1985). A still unknown trigger promotes the activation of these cells leading to a cascade of events resulting in the autoimmune destruction of beta-cells.

DCs are the most efficient antigen presenting cells (APCs). Immature DCs (imDCs) circulate in the periphery as sentinels. They have a low co-stimulatory capacity and low ability to present antigens, but they are very well equipped to capture antigens. They are able

to engulf pathogens by endocytosis, phagocytosis or pinocytosis (Svensson *et al.*, 1997; Sallusto *et al.*, 1995). This results in their maturation and migration to the lymphoid organs, spleen and lymph nodes where they will activate naive T cells. Once mature, DCs redistribute major histocompatibility complex (MHC) molecules to the surface and express high levels of co-stimulatory molecules (CD80/86) at their surface, which are essential for T cell stimulation (Jensen PE, 2007). Intracellular pathogens are cut into peptides in the cytosol and presented at the surface on MHC class I molecules which activates cytotoxic T cells (CD8⁺ T cells) (Banchereau *et al.*, 1998). Extracellular pathogens that are engulfed via the endocytic pathway, are processed and presented on MHC class II molecules resulting in T helper stimulation (CD4⁺ T cells) (Banchereau *et al.*, 1998). It has been shown that in certain circumstances, extracellular pathogens are presented on MHC class I to stimulate cytotoxic CD8⁺ T cells. This is a phenomenon called cross-presentation (Rock *et al.*, 2005). DCs are also important in the induction of tolerance (Banchereau *et al.*, 1998).

B cells develop and differentiate in the bone marrow. They are characterised by the expression of the B cell receptor (BCR) that recognises antigens presented by APCs (Delves, Roitt, 2000a). Upon antigen stimulation, B cells are clonally expanded and differentiated into antibody secreting plasma cells or memory B cells (Depoil *et al.*, 2008; Pierce, Liu, 2010). Antibodies released by the plasma cells are a soluble immunoglobulin copy of the B cell receptor that will help neutralise pathogens. B cells can be divided into different subsets depending in their degree of maturity (**Figure 1-4**) (LeBien, Tedder, 2008; Engel *et al.*, 2011). The lymphoid progenitor firstly differentiates into pro-B cells in the bone marrow. It is during the development into pro-B cells that immunoglobulin locus genetic rearrangements starts, but is not complete. Pro-B cells express only the Ig α , Ig β and calnexin that forms the pro-BCR. After differentiation, the pre-BCR is composed of the Ig μ chain that characterises the IgM molecule and the surrogate light chains. Once differentiation is accomplished, immature

B cells express a mature and functional BCR composed of assembled heavy and light chains, the IgM molecule (Delves, Roitt, 2000a). At this phase of development, B cells will undergo negative selection to avoid expansion of auto-reactive B cells. Cells that have successfully passed this checkpoint will then migrate to the periphery as transitional and intermediate B cells (Carsetti *et al.*, 2004). When they enter the SLOs, they are differentiated into mature follicular B cells, which results in expression of IgD at the cell surface (LeBien, 2000). B cell responses to antigen require the participation of T cells. When B cells have recognised an antigen, they migrate to the edges of the follicles in the SLOs; up-regulate expressions of CD40 and MHC class II and present antigens to T cells (Engel *et al.*, 2011). This step is crucial to trigger B cell proliferation and differentiation. At this stage, germinal centres (GC) are generated within the follicles. During the GC reaction, the B cell clones first differentiate into plasmablasts, which continue proliferating and start secreting low quantities of antibodies. Plasmablasts then differentiate into long-lived plasma cells and memory B cells. B cells can recognise other types of antigens i.e. lipids and nuclear acids without T cell help (Engel *et al.*, 2011). They can differentiate into short life plasma cells but are not capable of class switching or memory cell generation (Fagarasan, Honjo, 2000). However, they are essential for quick responses to pathogens.

Natural Killer cells (NKs) represent 10 to 15 % of the immune cell pool in the circulation. They share the same lymphoid precursor as other lymphocytes. NKs were first identified as “natural occurring killer lymphocytes with specificity for tumour cells” (Kiessling *et al.*, 1975). Detection of pathogenic antigens or stressed cells results in their activation, with the release of granules containing the perforin/granzyme machinery. Upon degranulation, perforin inserts itself in the membrane of the target cell forming a pore. This allows the entrance of granzyme, which will induce apoptosis of the target cell (Trapani, Smyth, 2002).

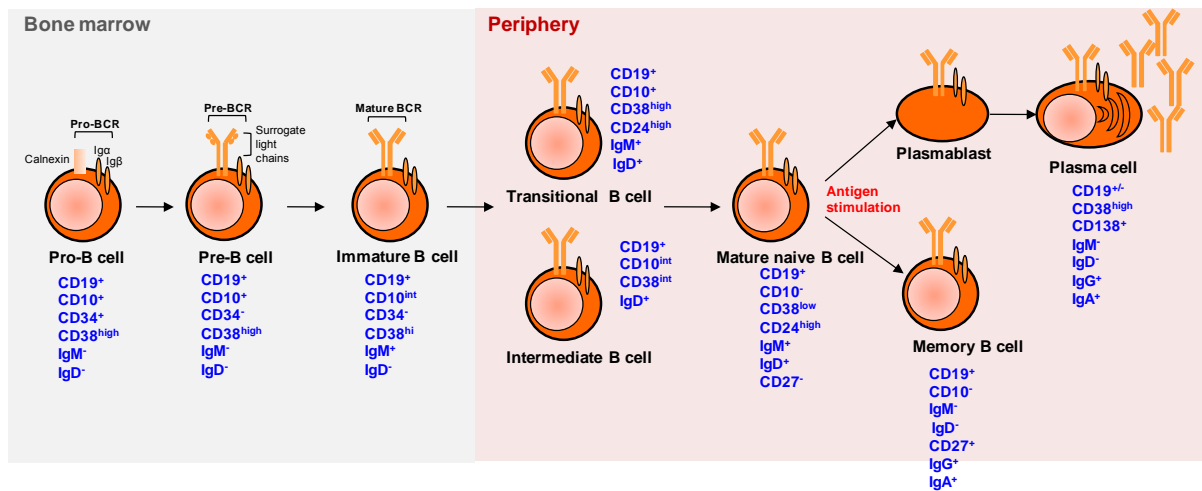


Figure 1-4: B cell development in humans

B cells develop in the bone marrow and enter the circulation as immature B cells to travel to SLOs where they differentiate into mature B cells. In SLOs, B cells meet with their antigen and become activated, leading to germinal centre formation. Activated mature B cells can differentiate into plasmablast, which begin to produce antibodies but are still dividing and then become antibody-secreting plasma cells. Some antigen-stimulated B cells become memory B cells. Differential expression of the numerous cell surface markers allow dissection of B cell development and recognition of the different subsets circulating in the blood.

NK cells can distinguish between a normal cell expressing MHC class I molecules and a stressed cell which has lost MHC class I, using receptors such as the natural killer cell immunoglobulin like receptor (KIMs). These inhibitory receptors allow the NK cells to inhibit NK activation if interacting with a healthy MHC class I bearing cell, and to specifically target stressed cells having no MHC class I, i.e. those that are a potential danger for the body. This mechanism is called “missing self recognition” (Vivier, 2006). NK cells are recognised by the expression of the CD56 marker. In humans, they can be divided into two populations, CD56^{low} (which also express CD16) and CD56^{high} NK cells subsets. These two populations differ in their trafficking properties. The most abundant type, CD56^{low}, is predominant in the blood, whereas CD56^{high} have greater capacity to migrate into lymphoid organs (Vivier, 2006). The latter do not have strong cytolytic activity, as they lack perforin. However, they do secrete high levels of IFN- γ in response to IL-12, IL-15 and IL-18 stimulation.

Finally, NK T cells are lymphocytes with characteristics close to cells from the adaptive immune system. They also have NK like properties, as they co-express a TCR/CD3 complex and CD56. They are able to rapidly kill target cells by using the perforin/granzyme machinery, and can also secrete cytokines such as IFN- γ , TNF- α and IL-4 in response to antigen stimulation. NK T cells do not recognise peptides presented by MHC molecules; instead they identify lipids presented on invariant CD1 molecules (Tupin *et al.*, 2007). There are different types of NK T cells. The most common are the invariant NK T cells that are CD1d-restricted. A lot of work has been conducted showing their capacity to aid in the clearance of bacteria, viruses and protozoan parasites.

In T1D, DCs can directly take up beta-cells' auto-antigens in the islets and migrate to the pancreatic lymph nodes where autoreactive CD8⁺ T cells and CD4⁺ effector T cells are stimulated upon recognition of the auto-antigens presented by the DCs (Yoon *et al.*, 2001; Morran *et al.*, 2008). These autoreactive T cells will then traffic to the islets where they will cause damage and recruit other cytotoxic cells (Yoon *et al.*, 2001; Morran *et al.*, 2008). CD8⁺ cytotoxic T cells can also directly recognise antigens presented on the up-regulated MHC class I on beta-cells (Notkins *et al.*, 2002). This results in up-regulation of the FasL/Fas apoptotic signal and perforin/ granzyme mediated cytotoxicity (Pirot *et al.*, 2008). Antigen presentation to CD4⁺ T cells is mediated by APCs presenting on MHC class II, thus resulting in production of cytokines (Mathis *et al.*, 2001). These cytokines can directly act on beta-cells or on the APCs that will in turn secrete more cytokines, nitric oxide or oxygen derived free radicals that induce cell death (Notkins *et al.*, 2001). The CD4⁺ effector T cells are also responsible for initiation of the activation of other cells such as B cells, resulting in the production of auto-antibodies (Morran *et al.*, 2008). Pro-inflammatory mediators such as IL-1, interferon-gamma (IFN- γ), tumor necrosis-alpha (TNF- α) (Induction of FasL/Fas pathway),

IL-2, IL-21, IL-18 and IL-17 produced by effector T cells can contribute to the suppression of beta-cells function and their subsequent apoptosis (Rabinovitch *et al.*, 2003; Emamaullee *et al.*, 2009). However, most of the current knowledge in this area comes from studies in murine models of T1D (Notkins *et al.*, 2001; Rabinovitch *et al.*, 2003). Indeed, the hazard in performing pancreatic biopsies is too high, therefore, what is known about the pathogenesis of human T1D comes from autopsy specimens, assays involving peripheral blood or isolated islets (Notkins *et al.*, 2001).

It is clear that inflammation can cause harm, in this case through the destruction of beta-cells. However, the immune system also has built into it, a system for self-regulation. Regulatory T cells (Treg) are key regulators of the immune function. Treg play a critical role in maintaining self-tolerance and controlling the extent of immune responses. They were first identified by the expression of the IL-2 receptor α chain, CD25 (Sakagushi *et al.*, 1995). However, CD25 is also a marker of activated T cells. Accurate definition of Treg came with the identification of the forkhead family transcription factor, FoxP3 specific to Treg (Hori *et al.*, 2003). Two types of Treg have been identified: natural Treg (nTreg) and induced Treg (iTreg). nTreg develop in the thymus and express a similar repertoire to naive T cells but are skewed to self-antigens, indicating their important role in tolerance (Pacholczyk *et al.*, 2008). iTreg conversely develop in the periphery upon conventional $CD4^+ CD25^-$ T cells activation. Consequently, they express a similar antigen repertoire to conventional T cells. Both Treg subsets exert their suppressor function upon antigen recognition (Pacholczyk *et al.*, 2008). However, once activated, Treg exhibit a T cell receptor (TCR)-independent bystander suppression of other T cells (Tang *et al.*, 2008).

Various mechanisms of Treg suppression have been determined, mainly using *in vitro* experiments. Treg can act either directly on effector T cells or indirectly as shown by APC absence or presence assays respectively. Direct mechanisms involve the consumption of IL-2

by Treg, secretion of anti-inflammatory cytokines such as IL-10 and TGF- β , induction of apoptosis through contact by granzymeB/perforin or galectin-1 action (Shevach *et al.*, 2009; Sojka *et al.*, 2008). Indirect action involves the action of Treg on APC by suppressing antigen presentation or modulation of co-stimulation involving CTLA-4, LAG-3, CD39 or Nrpl (Shevach *et al.*, 2009; Misra *et al.*, 2004; Liang *et al.*, 2008; Borsellino *et al.*, 2007; Sarris *et al.*, 2008).

It was first believed that Treg number was decreased in patients with T1D (Kukreja *et al.*, 2002). Subsequent studies have failed to demonstrate a Treg deficit, either in human T1D or in the Non Obese Diabetic (NOD) mice model of T1D (Brusko *et al.*, 2005; Lindley *et al.*, 2005; Putnam *et al.*, 2005; Brusko *et al.*, 2007).

Presence of Treg at sites of inflammation such as the pancreas of T1D patients (Bottazo *et al.*, 1985) suggests that their capacity to sense responses is not affected. Another possible mechanism to explain autoimmunity was shown to be caused by defect in Treg suppressive function (Lindley *et al.*, 2005). However this was not confirmed by other groups (Liu *et al.*, 2006). Finally, it is now believed that the deficit in Treg regulation is caused by a resistance of effector T cells to Treg suppression. This is mediated by alteration of sensitivity to transforming growth factor-beta (TGF- β) (Gregori *et al.*, 2003; You *et al.*, 2005; Lawson *et al.*, 2008; D'Alise *et al.*, 2008).

Defects in other regulatory mechanisms can potentially also contribute to the establishment of insulinitis. These relate to the control of T cell migration, or involvement of adipokines, both of which will be elaborated below.

1.3.2. Trafficking and recruitment of lymphocytes during inflammation

Inflammation is referred to the rapid and protective cascade of responses occurring in response to a local tissue injury, infection or occurrence of a local immune response (Goldsby *et al.*, 2001). It triggers the recruitment of leukocytes and humoral proteins required for

clearance of a pathogen or trauma (Ryan *et al.*, 1977). If successful, acute inflammation is resolved by restoring normal tissue homeostasis or formation of a connective tissue scar (Poher *et al.*, 2007; Springer, 1994). If the stimulus is not eliminated, the inflammation persists and evolves to become chronic inflammation that results in disease such as asthma, atherosclerosis, multiple sclerosis, rheumatoid arthritis and T1D (Poher *et al.*, 2007; Koulmanda *et al.*, 2007). Inflammation is characterised by four signs: pain, redness, swelling and heat (Springer, 1994). These result from the vasodilatation of blood vessels causing increased local blood flow and leakage of proteins into the tissues. This also acts on the local endothelium that adopts an inflammatory phenotype contributing to the recruitment of leukocytes (Poher *et al.*, 2007; Springer, 1994).

This thesis is primarily interested in understanding the modulation of inflammatory processes taking place in T1D. This includes the study of immune cell stimulation as well as their trafficking. Therefore it is important to understand how and where immune responses develop.

1.3.2.1. Localisation of immune responses

Once mature, T and B cells continuously traffic between the blood and the secondary lymphoid organs, a process called homing (von Andrian *et al.*, 2003; Cyster, 2003a). Secondary lymphoid organs (SLOs) are responsible for collecting antigens and facilitate their exposure to recirculating lymphocytes (Cyster, 2005). In addition, lymphoid organs provide a suitable environment for maintenance and development of B and T cells (Cyster, 2005). Secondary lymphoid tissues comprise the lymph nodes; which are clustered at sites such as the groin, axillae, neck and small intestine; the spleen, which collects blood-borne antigens, the mucosal lymphoid tissues of the gut (Peyer's patches), the nasal and respiratory tract, the urogenital tract and other mucosa. Lymph nodes are interconnected by lymphatic vessels,

which drain extracellular fluid (lymph) from tissues through the lymph nodes and back into the blood (Melody, 2001). This allows them to screen for antigens. All SLOs have a specific structure adapted to T and B cell activation (Itano *et al.*, 2003). The best example is the lymph nodes.

Within SLOs, B cells are concentrated in regions called follicles where they screen follicular DCs (Willard-Mack, 2006). Stimulation of B cells results in the formation of a germinal centre that will be referred as secondary follicles. Once differentiated into plasma cells, they migrate to the medullary cord where they will secrete antibodies released in the lymph (Shapiro-Shelef *et al.*, 2005).

T cells are located in the paracortical area where they survey DCs. T cell stimulation does not lead to germinal centre formation but causes enlargement of the paracortex. This compartmentation is possible only because of the combinatorial action of chemokines, integrins and selectins which guide the B and T cells to their respective zone (Cyster, 2003b; von Andrian *et al.*, 2003). Although chemokines are essential for T and B cells repositioning in the lymphoid tissues, it has been debated that this process was not caused by chemokines gradients as initially determined. In fact, T and B cell migration is governed by the "type" of High Endothelial Venules (HEV) (Miyasaka *et al.*, 2004). HEV are specialised for continuous capture and migration of recirculating lymphocytes (Miyasaka *et al.*, 2004). They display different recognition signals that allow selective entry of the cells in their homing compartment (Wei *et al.*, 2003). Homing of B cells to the follicle is guided by interaction of CXCR5 that is expressed on mature B cells, with CXCL13 expressed in the surroundings follicular stromal cells (Ansel *et al.*, 2000; Cyster *et al.*, 2005). T cells and DCs migration to the paracortex depends on CCR7 and its ligands CXCL19 and CXCL21 (Forster *et al.*, 1999; Gunn *et al.*, 1999). Both chemokines are made by paracortical stromal cells but also by HEV and DCs (Cyster, 1999). These characteristics of B and T cell homing differs in the spleen

lacking the HEV. Instead, B and T cells enter the spleen by terminal arterioles opening into the marginal sinuses or the red pulp (van Ewijk *et al.*, 1985).

In the periphery, the endothelium characteristics vary according their location in the different levels of the vascular tree and between different organs (Aird *et al.*, 2005). Thus, post-capillary venules of other tissues are for example specialised sites for recruitment of leukocytes following inflammation/infection insult. Other endothelium display differences in their expression profile of recruitment molecules but also phenotypic distinction such as fenestration in the islets (Henderson *et al.*, 1985).

1.3.2.2. The life of a T cell

T cells continuously traffic in the circulation through the secondary lymphoid organs to screen for antigens. In inflamed tissues, DCs take up antigens and bring them to the SLOs where they stimulate antigen-specific T cells (Jensen, 2007). Upon stimulation, naive T cells proliferate into effector T cells equipped to migrate to the site of inflammation/infection (Boes *et al.*, 2004).

Most lymphocytes fail to recognise their antigen and spend 12 to 18 hours for T cells and 24 hours for B cells, in the SLOs. After that, they recirculate in the blood either via the lymph or directly into the circulation (Cyster, 2003b). Lymphocytes spend only 30 minutes in the blood and then home back to (likely another) SLOs (Delves *et al.*, 2000). This cycle is repeated over and over again for several months (Cyster, 2005). Sequestration of T cells in the SLOs is crucial for the appropriate screening of antigens presented by DCs and is possible because of specific retention signals. Recent work has shown that CCR7 is not only required for T cell entry into SLOs but also for their retention and motility within the T cell zone (Pham *et al.*, 2008; Okada *et al.*, 2007). However, the CCR7 signal needs to be overcome to allow T cell egress from the lymphoid organs. This process is controlled by sphingosine-1-phosphate (S1P) (Mandala *et al.*, 2002).

- **Sphingosine-1-Phosphate (S1P) and T cell egress**

S1P is a sphingolipid derived from the phosphorylation of sphingosine in a reaction catalysed by two isoforms of the sphingosine kinase, SPHK1 and SPHK2 (**Figure 1-5a**) (Spiegel, Milstien, 2003). SPHK1 is located in the plasma membrane, whereas SPHK2 is in the mitochondria, endoplasmic reticulum and nucleus (Spiegel, Milstien, 2011).

As a bioactive compound S1P has a short half-life of 15 minutes (Venkataraman *et al.*, 2008) as it becomes rapidly dephosphorylated back to sphingosine and this makes it available for ceramide and sphingolipid synthesis. Alternatively, it is irreversibly cleaved by S1P lyase into its tran-2-hexadecenal and ethanolamine phosphate metabolites (Bandhuvula *et al.*, 2007) (**Figure 1-5b**).

The involvement of S1P in T cell egress from the lymph node was first revealed with the discovery of FTY720 as an immunosuppressive agent (Fujita *et al.*, 1996). FTY720 immunosuppresses through sequestration of T cells in SLOs (Mandala *et al.*, 2002). The fact that FTY720 shares structural analogy with S1P lead to its discovery as an agonist of four of the five S1P receptors, S1PR1, S1PR3, S1PR4 and S1PR5 (Brinkmann *et al.*, 2002). It was demonstrated that high levels of S1P cause T cell sequestration in the lymph nodes in the same way as FTY720 (Mandala *et al.*, 2002). This is consistent with the existence of a S1P gradient guiding T cells out of the SLOs, where S1P is low, to the circulation where S1P concentrations are high (**Figure 1-5c**). Measurement of S1P concentrations in the plasma is difficult because of its binding to albumin and high-density-lipoprotein (HDL) (Murata *et al.*, 2000). However, it has been generally accepted that S1P concentration in the plasma is in the micromolar range (Pappu *et al.*, 2007).

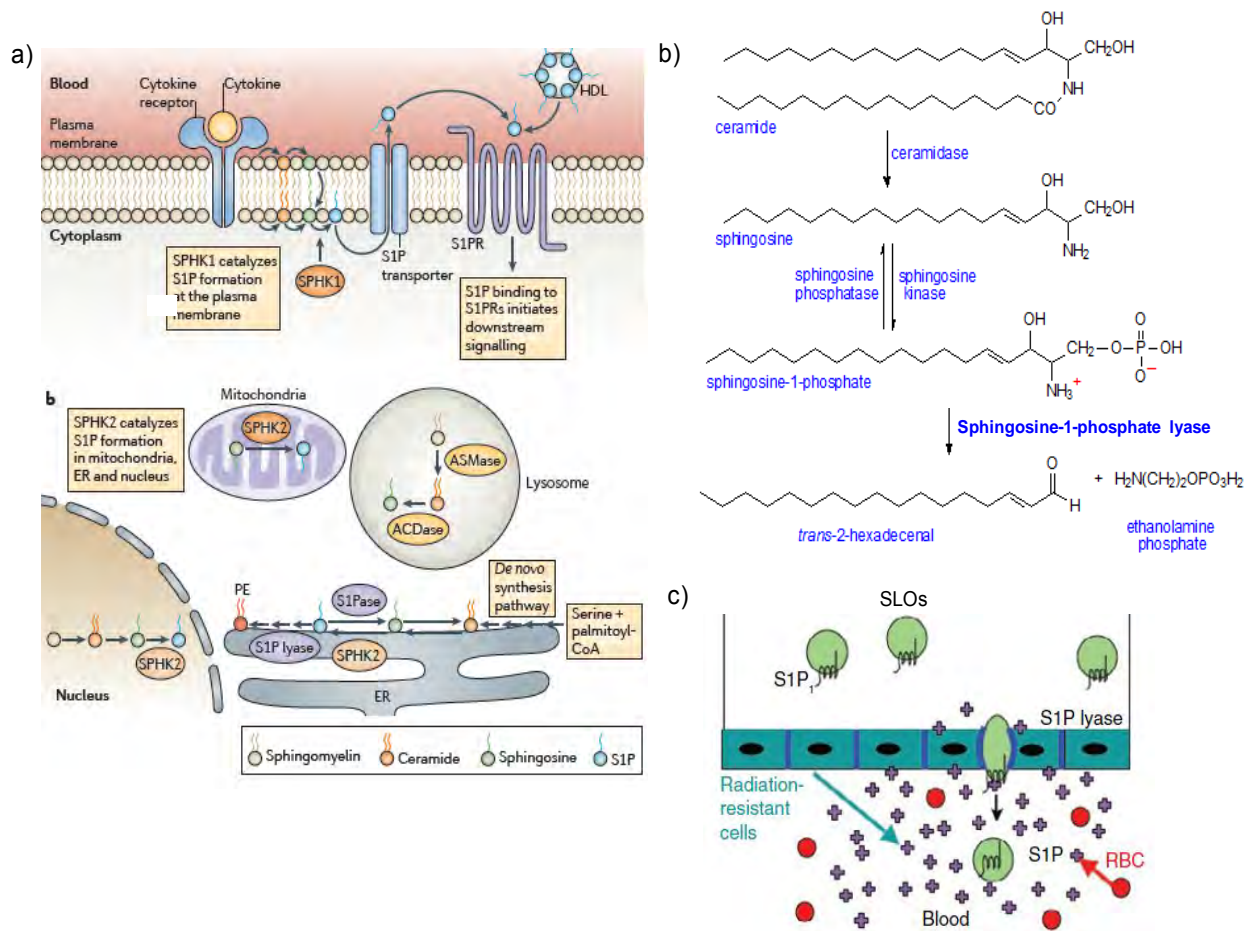


Figure 1-5: S1P and T cell migration

(a) S1P synthesis is catalysed by SPHK1 and SPHK2 (Spiegel *et al.*, 2011, copyright number: 2814670919041) and (b) S1P synthesis. SPHK1 located at the plasma membrane and SPHK2 at the endoplasmic reticulum (ER), mitochondria and nucleus, phosphorylate sphingosine. S1P produced in the ER can be degraded by the S1P lyase or dephosphorylated by the S1P phosphatase. At the membrane, S1P is exported through a transporter under the right cytokine stimuli. It then binds to high density lipoprotein (HDL) or albumin in the circulation and activates S1PR signalling. (c) In SLOs, S1P promotes the egress of T cell (green) to the lymph or blood where S1P is more abundant. S1P is produced by radiation-resistant cells such as endothelial cells (green squares) and red blood cells (RBC). After egress, T cells internalise the S1PR1 (S1P1) in response to high S1P levels (Schwab *et al.*, 2007, copyright number: 2814670695281).

In the lymph, S1P was measured in the nanomolar range as well as in the interstitial fluid of SLOs (Pappu *et al.*, 2007; Schwab *et al.*, 2005). S1P is produced by radiation-resistant cells (Pappu *et al.*, 2007) such as endothelial cells and red blood cells (Schwab *et al.*, 2005, Schwab *et al.*, 2007), which is consistent with the direction of the gradient.

Subsequent studies in S1PR1^{-/-} mice models have shown that the egress of T cells is dependent on S1PR1 (Matloubian *et al.*, 2004). Thus, lymphocytes lacking or with low level of S1PR1 cannot exit the lymph nodes. Then it was shown that naive T cell express high level S1PR1 when located in the lymph nodes which allow their recirculation. If the naive T cell meets with its antigen, activation results in decrease of S1PR1 expression. This allows the T cell to be sequestered in the lymph node in order to proliferate. Once the effector T cells are generated they up-regulate S1PR1 permitting the egress from lymph nodes to migrate to the site of inflammation/infection. Once in the circulation S1PR1 expression is down-regulated. The expression of S1PR1 enables T cells to sense the S1P gradient that drives T cells out of the lymph nodes while allowing the over-riding of the CCR7 mediated retention signals (Pham *et al.*, 2008).

S1P was for a long time considered as a promoter of T cell migration out of SLOs. However, recent evidence show that S1PR1 stimulation inhibit migration of lymphocytes from non-lymphoid peripheral tissues to afferent lymphatic vessels (Ledgerwood *et al.*, 2008), and this is clearly an area that requires further investigation.

- **The multistep adhesion cascade**

Effector and memory T cells are mobilised into the blood during an inflammatory response. Tissues are separated from the blood by the vascular endothelium constituted of endothelial cells (EC) that line all blood vessels. EC are tightly held together by junctions. They secrete an extracellular matrix called the basement membrane, which offer barriers and

anchors to EC (Nourshargh *et al.*, 2005). EC located in post-capillary venules display a wide range of adhesion molecules and chemokines allowing efficient recruitment of leukocytes to sites of inflammation/infection (Aird *et al.*, 2005). This process highly depends on the activation of EC by cytokines such as TNF- α , IFN- γ and IL-1 β , usually secreted following inflammation (Springer, 1995). The adhesion cascade to EC can be separated into eight steps (**Figure 1-6**):

- 1- Margination
- 2- Capture/ Rolling
- 3- Activation by chemokines
- 4- Integrin activation/ Arrest
- 5- Lipid induced activation
- 6- Migration over the endothelium
- 7- Trans-endothelial migration
- 8- Sub-endothelial migration

Initial capture to the endothelium is only possible because of a phenomenon called margination (Schmid-Schönbein *et al.*, 1980). This process represents the exit of free flowing leukocytes from the blood to tissues. The mechanism of margination involves the interaction of red blood cells with leukocytes flowing in the same vessel. Because the flow of erythrocytes is fast and localised down the centre of the vessels, it causes displacement of leukocytes towards the vessels wall (Goldsmith, Spain, 1984; Schmid-Schönbein *et al.*, 1980; Abbit, Nash, 2003). At this point, selectins or rolling receptors capture the lymphocytes allowing their rolling on the endothelium until encounter with the right inflammation signals that will firm their adhesion (Springer, 1995; Middleton *et al.*, 2002). There are three types of selectins which all have an N-terminal domain homologous to Ca²⁺ lectins: P-selectin, L-selectin and E-selectin. L-selectin is selectively expressed on almost all types of leukocytes

but on a subset of memory T cells (Springer, 1994; vonAndrian *et al.*, 1993). P-selectin was first discovered on platelets but has now been found on EC as well as E-selectin in inflamed conditions (Springer, 1994). It is now recognised that mainly P-selectin mediate T cell capture/rolling (Luscinskas *et al.*, 1995). E-selectin is crucial for adhesion of memory T cells (Picker *et al.*, 1991; Shimizu *et al.*, 1991). However, inhibition of P- and E-selectin on T cells induces a partial decrease of rolling (Luscinskas *et al.*, 1995). This has been explained by the evidence showing the capacity of $\alpha_4\beta_1$ (VLA-4-Very Late Antigen-4) integrins to support lymphocyte rolling on vascular cell adhesion protein-1 (VCAM-1) (Alon *et al.*, 1995; Berlin *et al.*, 1995; Johnston *et al.*, 1996; Luscinskas *et al.*, 1995).

After capture/rolling, T cells receive an activating signals through chemokines. Chemokines are small chemoattractant cytokines (8-16 kDa) subdivided into 4 families depending on the position of two highly conserved cysteines in the amino acid sequence: CXC, CX3C, CC or C. Inflamed EC up-regulate the expression of chemokines allowing downstream activation of integrins. Cinamon *et al.* determined the importance of CXCR4 binding to its ligand, CXCL12 (SDF-1) for lymphocyte migration (Cinamon *et al.*, 2001a; Cinamon *et al.*, 2001b). In inflamed conditions, IFN- γ production is likely to occur at the site of inflammation.

IFN- γ induces surface expression of the chemokines CXCL9 (Mig), CXCL10 (IP-10) and CXCL11 (I-TAC) on the endothelium (Mazanet *et al.*, 2000; Piali *et al.*, 1998). These chemokines specifically bind to CXCR3, a G-protein-coupled receptor (GPCR) chemokine receptor highly expressed on effector and memory T cells (McGettrick *et al.*, 2009; Piali *et al.*, 1998). Inhibition of CXCR3 signalling reduces T cell adhesion on IFN- γ /TNF- α stimulated EC indicating its importance in firm adhesion of T cell (Piali *et al.*, 1998; Curbishley *et al.*, 2005). However, this is only possible if there is a local production of IFN- γ .

Chemokines binding to their receptor induces integrins activation by triggering a complex intracellular signalling cascade within milliseconds (Ley *et al.*, 2007). This process is identified as “inside-out signalling” that increases the affinity of integrins, by extension of the tail and their valency on the T cell surface resulting in binding to their ligands on the endothelium (Ley *et al.*, 2007; Hogg *et al.*, 2011). For T cell migration, this is mainly mediated by CXCR3 signalling that activates LFA-1 ($\alpha_L\beta_2$ -Lymphocyte function-associated antigen-1) binding to ICAM-1 and VLA-4 ($\alpha_4\beta_1$) binding to VCAM on EC as inhibition of these suppress T cell adhesion *in vivo* and *in vitro* (Johnston *et al.*, 1996; Johnston *et al.*, 2000; Alon *et al.*, 1995).

For a long time, chemokine signals and re-organisation of the actin cytoskeleton during spreading of the EC were believed to be sufficient to support T cell transmigration. However, recent evidence demonstrates the importance of prostaglandin D2 (PGD2) in this process (Ahmed *et al.*, 2011). In this study, antagonism of the PGD2 receptor (DP-2, CRTH2) on memory T cells resulted in the blockade of transmigration, but not adhesion. PGD2, a member of the eicosanoid family and is highly produced by mast cells. During allergy and asthma it is released in excessive quantities causing vasodilatation, flushing, hypotension and syncopal episodes (Roberts, Sweetman, 1985). A similar process involving PGD2 was found for neutrophil transmigration via action on the DP-1 receptor (Tull *et al.*, 2009). Whilst the mechanism underlying PGD2 action is not fully characterised, it may involve an action on CCR7 for migration across lymphatic EC (Ahmed *et al.*, 2011).

After attachment, T cells migrate on the surface (Middleton *et al.*, 2002). This mechanism involves polarisation of T cells. Actin polymerisation at the front results in creation of a leading edge and the formation of a uropod at the back through actin-myosin based contraction (Soriano *et al.*, 2011). This causes detachment at the back and attachment at the front (Kinashi, 2005).

Until recently, it was believed that T cells could transmigrate through endothelium only by using a paracellular route, located at intercellular junctions. However, it is now clear that T cells can also directly cross the endothelium using a transcellular route, both ways involving different mechanism and molecules. The paracellular path involves molecules such as CD31, CD99, PECAM1, ICAM-2, LFA-1, JAM-A, B, C and VE-cadherin that accumulate at the extravasation site (Ley *et al.*, 2007; Muller, 2003). The intracellular route works through ligation of ICAM-1 resulting in its internalisation into actin- and caveolae-rich regions. These link together and form an intracellular channel referred as vesiculo-vacuolar organelles, permitting the migration of the lymphocytes (Ley *et al.*, 2007; Millan *et al.*, 2006).

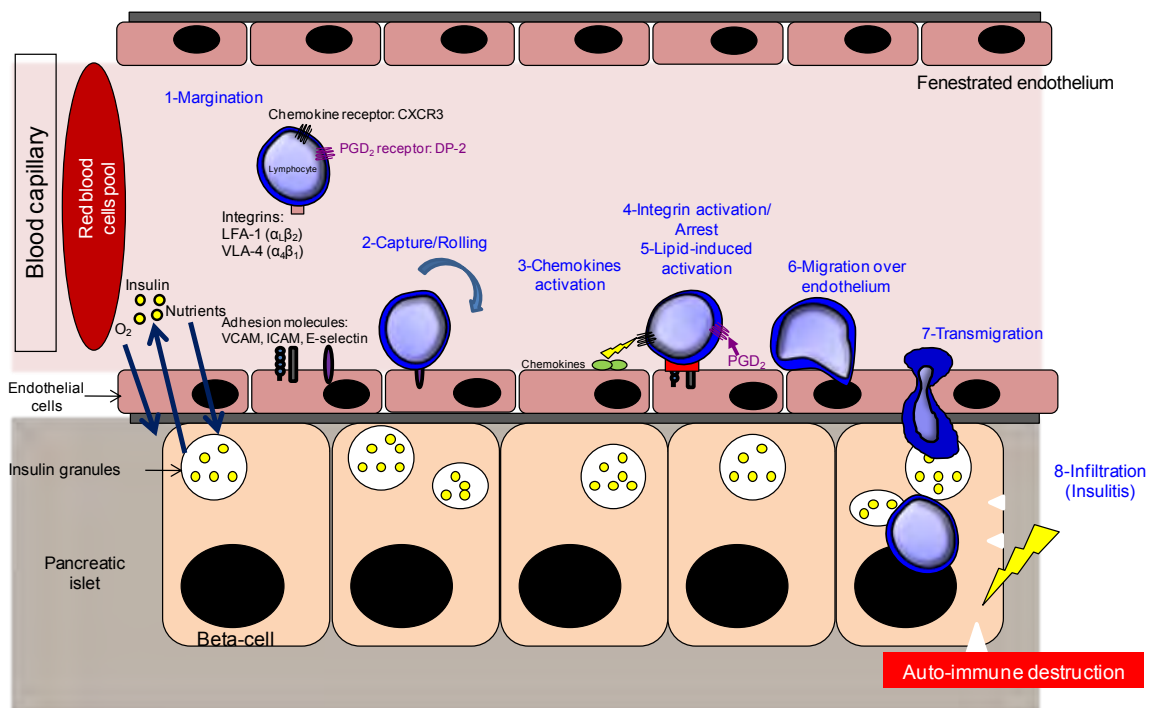


Figure 1-6: Schematic representation of the adhesion cascade leading to insulinitis in T1D Insulin circulates through endothelial cells from the beta-cells into the blood circulation. Endothelial cells are also important for the transfer of nutrients and oxygen to beta-cells. However, auto-reactive T cells, activated in the pancreatic lymph nodes, are recruited by chemotaxis to the islets and can cross the endothelial cells after rolling, by interacting with adhesion markers such as ICAM, VCAM and E-selectin. Autoreactive T cells then migrate into the islets to recognise auto-antigens and cause the auto-immune destruction of beta-cells.

1.3.3. Vascular function in T1D

In T1D, islet specific T cells are activated in the draining pancreatic lymph nodes following presentation of auto-antigens (Hoglund *et al.*, 1999). They then traffic to the islets via the islet microcirculation. The islet microcirculation is characterised by a dense network of capillaries lined by a distinctive fenestrated endothelium (Vajkoczy *et al.*, 1995; Olsson *et al.*, 2006). High vascularisation of the islets is crucial for nutrient, oxygen and insulin transfer between beta-cells and the circulation. This is allowed by the unique structure of the islet. Each beta-cell is located no more than one cell away from a capillary and the endothelial fenestration allows small molecules to quickly diffuse through the small pores in the endothelium (Henderson, Moss, 1985). However, islet endothelial cells also represent the portal of entry of auto-reactive T cells in T1D (**Figure 1-6**). Islet endothelial cells express common endothelial markers such as von Willebrand factor, CD31, ICAM-1, VCAM-1 and E-Selectin (Lozanoska-Ochser *et al.*, 2008).

Studies in diabetic mice models and pancreatic biopsies of human islet from T1D patients show that islet endothelial cells in T1D adopt an inflammatory phenotype. Indeed, the expression of ICAM-1, VCAM-1 and E-selectin, is increased on islet endothelium from mouse model of T1D and in patient (Hanninen *et al.*, 1992; Hanninen *et al.*, 1993; Rasmussen *et al.*, 2002; Xie *et al.*, 2008). These molecules facilitate the adhesion and transmigration of auto-reactive T cells from the blood to the inflamed pancreas via interaction with their corresponding ligands on the T cell surface. These ligands, such as LFA-1 ($\alpha_L\beta_2$) and VLA-4 ($\alpha_4\beta_1$), are also increased on activated T cells and monocytes in T1D (Xie *et al.*, 2008; Shimizu *et al.*, 1991). The expression of adhesion molecules on both endothelial cells and lymphocytes is modulated by cytokines (Ding *et al.*, 2000). Islet endothelial cells also express high levels of MHC class I and II, which are up-regulated during inflammation characterising T1D (Itoh *et al.*, 1993; Somoza *et al.*, 1994). Co-stimulatory molecules such as CD86 and

ICOS-L are also expressed on islet endothelial cells (Lozanoska-Ochser *et al.*, 2008). The presence of presenting and co-stimulatory molecules confirms the involvement of islet EC in the progression of insulinitis. Indeed, due to the inflammatory milieu, islets EC presentation of auto-antigens such as GAD65 activates and facilitates the migration of GAD65 specific auto-reactive T cells into the islets (Greening *et al.*, 2003; Marelli-Berg *et al.*, 1999). Blocking of PSGL-1 in mice attenuates incidence of T1D (Huang *et al.*, 2005). T cell transmigration through the islet EC is increased by activation of integrins either by TCR-mediated change of LFA-1 ($\alpha_L\beta_2$) avidity or by chemokine signalling emphasis (Katagiri *et al.*, 2003). A number of chemokines are associated with the development of T1D. Interestingly, CXCL10 circulating levels are higher in newly diagnosed patients (Shimada *et al.*, 2001). As mentioned earlier, CXCL10 is crucial for T cell migration and binds to CXCR3 highly expressed on effector and memory T cells. It has been found to be produced by beta-cells at high levels, resulting in excessive recruitment of T cells to the islets (Frigerio *et al.*, 2002, Rhode *et al.*, 2005). CXCL10 blockade prevents diabetes in mice by blocking T cell infiltration in the pancreas (Frigerio *et al.*, 2002; Cristen *et al.*, 2003). The CCL21-CCR7 interaction is also involved in the emigration of auto-reactive T cells in the islets (Savinov, Burn, 2010). CCL21 is also expressed in the islet region, especially in the inflamed pancreatic lymphatics (Qu *et al.*, 2005). CCL21 is essential to facilitate the recruitment of auto-reactive T cells in the pancreatic lymph nodes where they meet with their auto-antigen (Savinov *et al.*, 2003).

These examples whilst reflecting the complexity behind the control of autoreactive T cell migration, also reveals potential target to stop its progression. However, the implications of such a variety of mechanisms imply that targeting single pathways may not be efficient for therapy. In addition, it is now recognised that development of T1D is influenced not only by genetic factors that predispose to immune defects but also the environment (Furlanos *et al.*, 2008; Wilkin, 2001) (Chapter 1-section 2). The line of research in this PhD originated from a

basic hypothesis built on a recent understanding of environmental contributions to the development of T1D.

2. T1D, obesity and insulin resistance

Genes associated with risk in humans include HLA complex especially HLA-DR and HLA-DQ, CTLA-4, PTPN22 and IL-2RA (Concannon *et al.*, 2005; Nejentsev *et al.*, 2007; Concannon *et al.*, 2009). As mentioned before (Chapter 1-section 1.2), the prevalence of diabetes has been rising for the last decade. This rapidity suggests a role for the environment. This is supported by evidence that T1D subject's frequency with high risk HLA-DR genotypes has decreased over the last decades (Hermann *et al.*, 2003a and b). Possible environmental triggers include viral infection, vaccination, toxins, increasing obesity and insulin resistance (IR) (Knip *et al.*, 2005; Pang, Narendran, 2008).

IR is a condition in which the bioactivity of insulin is decreased. This results in increased requirement of insulin to maintain normal blood glucose levels. IR is generally caused by weight gain and physical inactivity. It can be measured by different methods. Measurement of fasting insulin is the most convenient way but not the most accurate. The Homeostasis Model Assessment (HOMA) is another simple way to measure IR. This model will mathematically estimate insulin sensitivity (HOMA %S) and level of beta-cell function (HOMA %B) by direct measurement of fasting plasma glucose and fasting plasma insulin (Matthews *et al.*, 1985). The most accurate way of measuring IR in patients with T1D, is the euglycaemic hyperinsulinaemic clamp; however, this test is more complicated (DeFronzo *et al.*, 1979, Pang, Narendran, 2008). A constant dose of insulin (40mU/min/m²) is continuously administrated to the subject. Glucose is also administrated, and the clamp test consists in measuring how much glucose is required to maintain the plasma blood glucose levels constant, this reflecting the whole body glucose disposal rate (GDR) capacity. A low GDR is

associated with insulin resistance. The GDR can also be estimated in subjects with T1D (eGDR), using HbA1c, blood pressure (HTN) and Waist/Hip ratio (WHR). With this method the estimated GDR can be calculated by the following equation: $24.31 - 12.22 \text{WHR} - 3.29 \text{HTN} - 0.57 \text{HbA1c}$. This score correlates well with clamp-derived values in adult T1D subjects (Williams *et al.*, 2000). The estimated GDR appears to be the easiest way to predict IR in T1D and has now been used in a number of studies (Orchard *et al.*, 2003).

Interestingly, about 26% of patients with T1D are insulin resistant (McGill *et al.*, 2008). Recently, three surveys on pre-T1D patients in Australia (Furlanos *et al.*, 2004), United States (Xu P *et al.*, 2007) and Europe (Bingley PJ *et al.*, 2008) suggest that IR is associated with the accelerated development of T1D. Obesity is a common cause of IR and its incidence has been dramatically increased in the last 10 years (James *et al.*, 2004). Previous reports have shown that people who develop T1D are heavier in early childhood than healthy controls (Baum *et al.*, 1975; Johansson *et al.*, 1994; Hypponen *et al.*, 1999; Bruining *et al.*, 2000). In addition, fatter children develop T1D at a younger age in some studies (Kibirige *et al.*, 2003). Therefore, obesity, IR and T1D may be related, and this may provide an explanation for the rise in T1D. However, how does IR influence the rate of development of T1D?

IR may lead to beta-cell stress, and this may invite autoimmunity, resulting in early development of T1D (Dahlquist, 2006). Other suggestions involve direct influence of IR on islet autoimmunity. Direct beta-cell apoptosis can be induced by glucotoxicity and lipotoxicity both of which are associated with IR. This also increases antigen presentation by beta-cell, causing acceleration of auto-immunity (Bjork *et al.*, 1992; Trudeau *et al.*, 2000). Other mechanisms involve an effect of the adipose tissue that has been shown to regulate T cell immunity. Indeed, fat accumulation associated with IR, induces a shift in the balance of

adipose tissue resident T cells (Nishimura *et al.*, 2009; Winer *et al.*, 2009, Feuerer *et al.*, 2009).

I am interested in the effect of adipose tissue derived cytokines (adipokines) on immunity, and whether this may mediate the association between IR and T1D, and the accelerated incidence of T1D that has been described in epidemiological studies.

3. Adipokines

3.1. Leptin

3.1.1. Pleiotropic properties of leptin

Leptin is a 16kDa peptidic hormone, a product of the *ob* gene located on chromosome 7, secreted mainly by white adipocytes in proportion to the body fat mass (Zhang *et al.*, 1994; Geffroy *et al.*, 1995). Leptin is composed of four α -helix and two β -sheets similarly to the structure of the IL-6 family of cytokines (Zhang *et al.*, 1997). The leptin receptor, a product of the *db* gene, is a member of the class I cytokines receptors family including gp-130 and IL-6 receptor (Tartaglia *et al.*, 1995). By alternative splicing, at least 5 different transcripts, named ObR-a to ObR-e, are produced from the *db* gene in mice. In humans, only the ObR-a, b, e and another poorly characterised variant, not present in mice, are so far characterised (**Figure 1-7a**). The different isoforms have in common the extracellular part, the transmembrane domain, apart from ObR-e which is soluble, but have a variable intracellular domain. All isoforms but ObR-e contain the JAK (Janus-family tyrosine kinase) binding Box 1 motif. However, only ObR-b displays the four tyrosine residues (Tyr974, Tyr985, Tyr1077 and Tyr1138) required for efficient signalling. Once phosphorylated, the tyrosines represent docking sites for signalling proteins containing SH2 domains, involved in the JAK/Stat signalling pathway (Chen *et al.*, 1996; Lee *et al.*, 1996; Cioffi *et al.*, 1996). Although, JAK/Stat signalling is recognised as the main pathway activated by leptin, the MAPK

(Mitogen-activated protein kinase) cascade is also involved in the leptin signalling (**Figure 1-7b**). Studies have shown that despite lack of the four tyrosines, OBR-a is able to transmit signals, although substantially weaker than OBR-b.

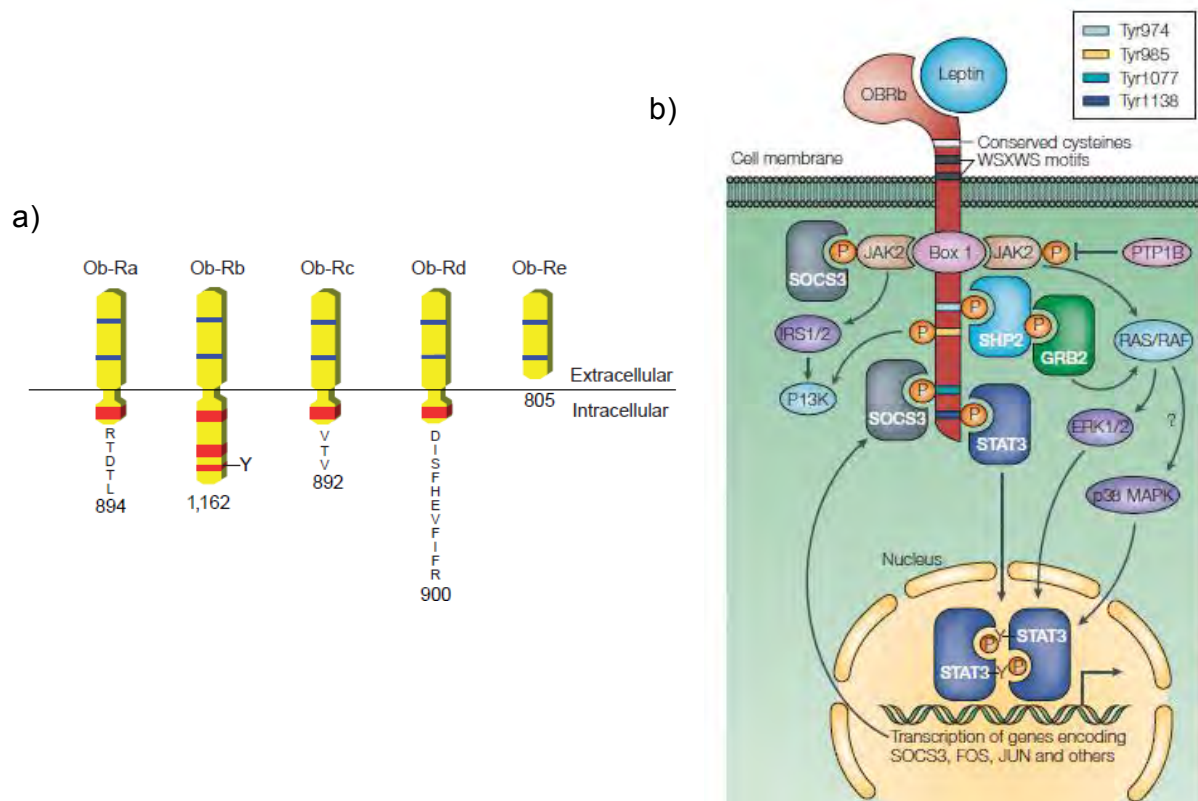


Figure 1-7: Leptin receptors and their signalling pathways in mice and humans

(a) Leptin receptor isoforms in mice and humans showing the conserved cysteines and motifs in the extracellular region (blue) and Box 1 present on all isoforms but the soluble form and the two sets of tyrosine in the intracellular region that are specific to *LEPR* (ObR-b) (Friedman *et al.*, 1998; copyright license number: 2817160481320). (b) *LEPR* (ObR-b) signalling involves the JAK/STAT pathway but can also trigger the MAPK cascade (LaCava *et al.*, 2004; copyright license number: 2817160655753).

However, the pathway activated by OBR-a is controversial. Studies have reported activation of both MAPK and JAK/Stat pathways (Bjorbaek *et al.*, 1997; Yamashita *et al.*, 1998). ObR-a has been involved in regulation of leptin transport into the brain (Hileman *et al.*, 2002). Finally, ObR-e is a soluble form of the leptin receptor. It circulates in the blood where it binds to leptin and consequently prevents its degradation and clearance (Huang *et al.*, 2000).

Leptin regulates body weight through the control of appetite and metabolism (Friedman *et al.*, 1998). The metabolic effects of leptin are mediated through its interaction with *LEPR* in the hypothalamus region, which is involved in the regulation of the appetite (Mercer *et al.*, 1996). Leptin deficient mice (*ob/ob*) or leptin receptor deficient mice (*db/db*) have a severe obesity phenotype characterised by an increase in food intake and decrease in energy expenditure. Leptin administration to these mice restores the obesity phenotype (Pelley *et al.*, 1995). Leptin receptors expression has also been detected in peripheral tissues such as adrenal gland (Tena-Sempere *et al.*, 2000), placenta (Senaris *et al.*, 1997), stomach (Mixe *et al.*, 2000), ovary (Karlsson *et al.*, 1997), muscles, heart, kidneys, adipocytes (Kielar *et al.*, 1998), liver, pancreatic beta-cells and immune cells (Friedman *et al.*, 1998; Zhang *et al.*, 2005). As a result, leptin has the potential to influence reproduction (Chehab *et al.*, 1996), insulin secretion (Friedman *et al.*, 1998), haematopoiesis (Benett *et al.*, 1996), angiogenesis (Park *et al.*, 2001), metabolism of bones (Ducy *et al.*, 2000), lipids and glucose (Friedman *et al.*, 1998), and both the innate and adaptive immune response (Lord *et al.*, 1998, Sanchez-Margalet *et al.*, 2003, Matarese *et al.*, 2004).

3.1.2. *Leptin in immunity*

3.1.2.1. *Leptin modulates innate and adaptive immunity*

Although a major role of leptin is to regulate body weight, leptin is now recognised to have important effects on both innate and adaptive immune responses (**Figure 1-8**). A number of studies have observed immune abnormalities in *ob/ob* and *db/db* mice (Boillot *et al.*, 1986; Palmer *et al.*, 2006; Papathanassoglou *et al.*, 2006). Firstly, thymic reduction and consequently decrease in T cells number and their precursors is observed in *ob/ob* or *db/db* mice (Howard *et al.*, 1999). In these animals, T cells responses are not efficiently stimulated because of the concomitant decrease in APCs functions. In humans, leptin deficiency is also

associated with obesity and lymphopenia (Farooqi *et al.*, 2002). The studies in leptin deficiency models indicate the important role of leptin as a pro-inflammatory cytokine.

Leptin was reported to dose-dependently enhance the proliferation of peripheral blood mononuclear cells (PBMC) and highly purified CD4⁺ T cells, both co-stimulated with an allogenic stimulator (Lord *et al.*, 1998). Leptin does not enhance the proliferation of *db/db* mice allogenic splenocytes even at high concentration (100nM), showing that the leptin effect is specific to leptin receptor signalling on CD4⁺ T cells. This article also showed that the T cell production of IL-2 and IFN- γ is leptin dose-dependent. Other studies have shown a similar effect of leptin from 0nM to 100nM specifically on CD4⁺ and CD8⁺ T cells stimulated with phytohaemagglutinin (PHA) or concanavalin A (Martin-Romero *et al.*, 2000). In addition to its action on proliferation, leptin seems to skew T cell responses to T helper (Th1) by inhibiting differentiation to T helper 2 (Th2) (La Cava *et al.*, 2004). Another important effect is the ability of leptin to inhibit T cell apoptosis by down-regulation of the Fas and Bcl-2 pathways. This has also been observed on B cells in addition to an increased production of IgG2a and TNF- α , IL-6 and IL-10 (Papathanassoglou *et al.*, 2006; Agrawal *et al.*, 2011; Lam *et al.*, 2010).

On the other hand, other reports have noticed that leptin can suppress peripheral lymphocyte function when administrated by intracerebroventricular injection. It appears that in contrast to its direct activating effect, leptin is able to suppress lymphocytes function indirectly through the activation of the sympathetic nervous system (Okamoto *et al.*, 2000).

This new paradoxical suppressing effect of leptin has been attributed to an interesting heterogeneity of cellular behaviour between naive and memory T cells which were differentially affected by leptin (Lord *et al.*, 2002). It appears that leptin inhibits the anti-CD3-driven proliferation of memory T cells, whereas the naive CD3⁺ T cells proliferation was enhanced in co-stimulation conditions.

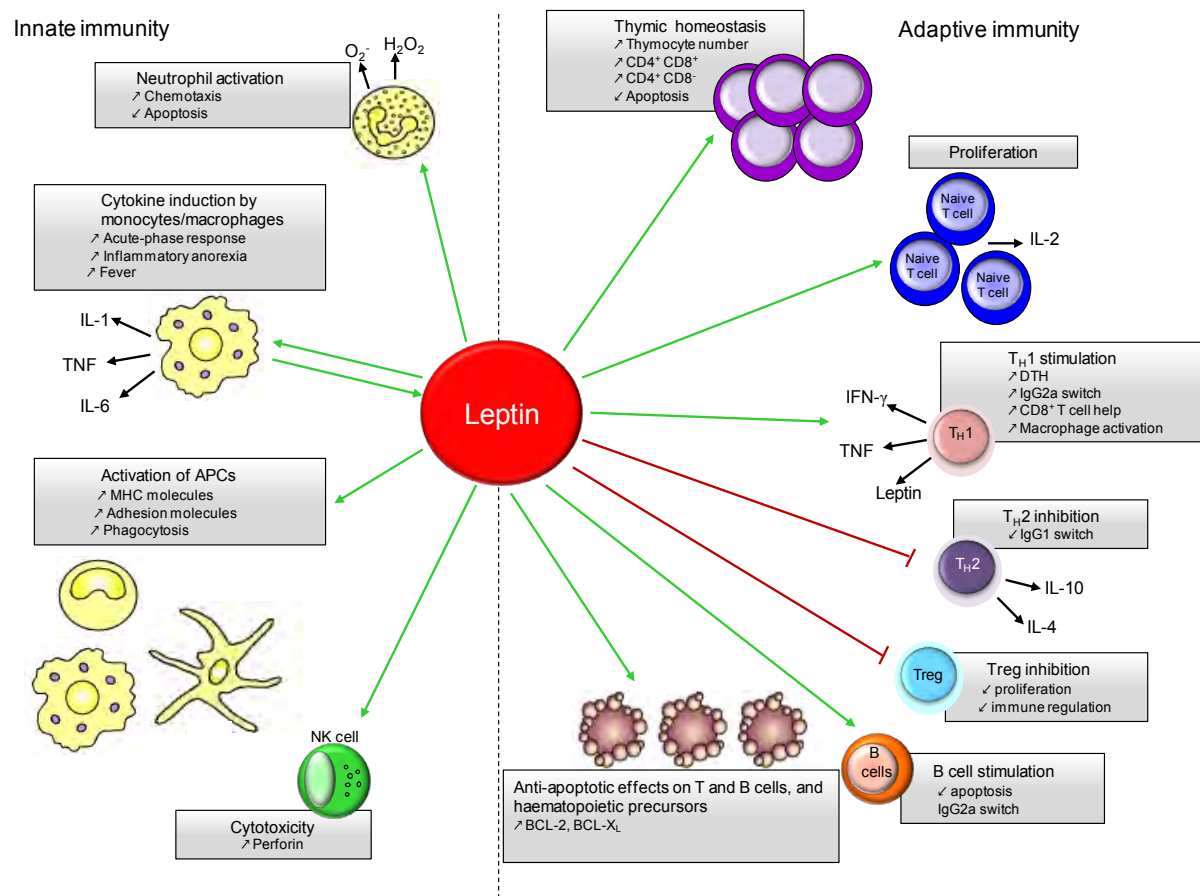


Figure 1-8: Leptin and immunity

Leptin can influence both innate and adaptive immunity. In innate immunity, leptin modulates neutrophil activation as well as monocytes and macrophages capacity to produce cytokines. Leptin can also increase APCs phenotype and function, in addition to cytotoxicity of NK cells. In the adaptive immunity, leptin has direct effects on thymic homeostasis and T cell proliferation. Leptin can skew immune responses towards a Th1 phenotype and inhibit Th2 responses as well as Treg proliferation and their immune-regulation capacities. Leptin can also stimulate B cell proliferation and reduce apoptosis of T and B cells. In conclusion, leptin has several pro-inflammatory actions on the immune system (Adapted from LaCava *et al.*, 2004).

However, in these conditions there is still an important production of pro-inflammatory cytokines. These findings show further complexity in the action of leptin on the immune system.

A few years later, the same group reported that leptin can also act as a negative signal for the proliferation of human FoxP3⁺CD4⁺CD25⁺ Treg *in vitro* (DeRosa *et al.*, 2007). In this study, the authors also reported that Treg produce leptin. Anti-leptin antibody blockade restores Treg proliferation during CD3/CD28 bead stimulation *in vitro*. This effect is also noticed *in vivo* when Treg from normal mice are transferred into leptin-deficient mice resulting in Treg proliferation and expansion. Treg from *db/db* mice show higher proliferation *in vitro* than Treg from normal mice, suggesting that the effect of leptin is specific to leptin receptor signalling. Interestingly, Treg from NOD mice deficient for the leptin receptor, proliferate more than Treg from the normal NOD mice in response to antigen-specific stimulation with GAD65. In these conditions, T effector cells proliferation is reduced. These findings are consistent with the potential involvement of leptin in autoimmunity in T1D because Treg are an important regulator of autoimmunity.

Leptin also modulate innate immunity. LEPR is highly expressed on neutrophils, resulting in down-regulation of apoptosis, increased activation and chemotaxis (Bruno *et al.*, 2005; Cadelfie-Chezet *et al.*, 2003). Leptin promotes the activation of phagocytosis by monocytes/macrophages, their proliferation, the secretion of pro-inflammatory cytokines (TNF- α , IL-1, IL-6) and nitric oxide without preliminary co-stimulation with PHA (Zarkesh-Esfahani *et al.*, 2001; Santos-Alvarez *et al.*, 1999). MHC molecules expression is up-regulated on APCs as well as co-stimulatory and adhesion molecule (Mattioli *et al.*, 2005). On natural killer (NK) cells, leptin induces an increase in perforin production (Zhao *et al.*, 2003).

3.1.2.2. *Leptin and migration of immune cells*

There are a number of studies of leptin action on inflammatory cell migration. Leptin stimulates chemotaxis of neutrophils, monocytes, macrophages and DCs (Cadelfie-Chezet *et al.*, 2003; Gruen *et al.*, 2007; Mattiolo *et al.*, 2005). In monocytes and macrophages, leptin acts as a chemoattractant by increasing calcium influx as well as the activation of the Janus kinase (JAK) and the phosphatidylinositol 3-kinase (PI3K) signalling pathways (Gruen *et al.*, 2007). However, the mechanism underlying leptin effects are varied. In DCs, studies reported that leptin intensifies DCs migration by both favouring cytoskeleton dynamics and up-regulating CCR7 surface expression (Mattiolo *et al.*, 2005).

3.1.3. *Leptin in T1D*

3.1.3.1. *Circulating levels*

Data regarding plasma leptin levels in T1D are conflicting (**Table 1-1**). Most reports including patients with long-duration of T1D have shown higher circulating leptin concentration compared to healthy controls (HC) (Kiess *et al.*, 1998; Luna *et al.*, 1999; Hanaki *et al.*, 1999; Fluck *et al.*, 1999; Kirel *et al.*, 2000; McCormick *et al.*, 2001; Soliman *et al.*, 2002; Bideci *et al.*, 2002; Sandoval *et al.*, 2003; Lo *et al.*, 2004; Gilliam *et al.*, 2006). Generally, insulin therapy induces an increase in circulating leptin levels in T1D, suggesting that leptin secretion is controlled by insulin (Bideci *et al.*, 2002; Soliman *et al.*, 2002; Kiess *et al.*, 1998). However, some reports have shown no effects of insulin (Luna *et al.*, 1999; Kirel *et al.*, 2000; McCormick *et al.*, 2001). Adding more controversy, some studies have shown no differences or even lower leptin levels between T1D and HC before or after insulin therapy. These studies seem to mainly involve newly diagnosed or pre-diabetes patients (Lo *et al.*, 2004; Hanaki *et al.*, 1999; Gilliam *et al.*, 2006). From these studies, we can conclude that

leptin levels are lower or similar to HC in pre and newly T1D. After insulin treatment, leptin levels rise which explain the high levels observed in long-duration of diabetes patients.

Measuring circulating levels is complex. First, leptin levels follow a diurnal cycle and change with food intake. Leptin in circulation can also be bound to its soluble receptor isoform (Tu *et al.*, 2008). The proportion of leptin-bound is not defined and causes variations in leptin measurement. Another factor influencing leptin levels is puberty. Indeed, studies have shown elevation of circulating leptin levels from pre-puberty to puberty to adulthood in healthy or diabetic or obese patients (Verrotti *et al.*, 1998; Ahmed *et al.*, 2001). For this reasons measurement and interpretation of leptin levels in patients versus controls should be done cautiously, and these differences may explain discrepancies between reports in this area.

Study	n HC	n T1D	Mean age T1D (years)	Mean BMI T1D (kg/m ²)	Mean HbA1c (%)	T1D duration (months)	Mean leptin T1D (ng/ml)	Mean leptin HC (ng/ml)	Effect of insulin therapy on leptin levels in T1D
Bideci <i>et al.</i> , 2002	12	16	13±2.6	21.2±2.7	10.2±1.9	80±31	19.1±7.6	6.1±2.9	increased
Sandoval <i>et al.</i> , 2003	15	16	28±3	27±2	8.4±0.7	NA	13.8±3	5.4±1	NA
Luna <i>et al.</i> , 1999	66	43	10±0.6	17.5±0.4	10.5±0.3	4.8 years±0.8	8.6±1.0	4.1±0.4	no effect
Kirel <i>et al.</i> , 2000	35	35	12.7±3.4	18.1±2.5	11.9±3.5	35±45	0.83±0.3	1.5±0.94	no effect
Soliman <i>et al.</i> , 2002	10	45	7.9±1.5	17.2±1.7	8.1±1.8	Newly vs 3.5 years ±2.5	Newly: 1.1±0.8 T1D long: 2.1±1.4 *	1.3±1.5	increased
Kiess <i>et al.</i> , 1998	710	13 newly 134 T1D long	9.8±3.7	15.2±3	10±1.9	NA	Newly: 1.28±1.6 T1D long: 5.18±5.48 *	2.2	increased
McCormick <i>et al.</i> , 2001	NA	17	8.6±4.9	15.5±1.8	10.9±1.6	Newly	Newly: 4.3±1.1 Insul in treated: 5.1±3.3 ^{ns}	NA	No effect
Lo <i>et al.</i> , 2004	33	58	10.98±4.61	19.11±2.73	8.70±1.59	Newly	5.19±3.37 ^{ns}	5.03±3.36	NA
Hanaki <i>et al.</i> , 1999	19	19	9.8±1	16.8±0.5	11.6±0.6	Newly	3.3±0.2 *	6.2±0.9	increased time dependently
Fluck <i>et al.</i> , 1999	NA	28	8.75±4.05	15.79±2.47	11.3±1.9	Newly	1.37±0.56	NA	increased time dependently
Gilliam <i>et al.</i> , 2006	33	26	26.4	24.9	NA	Pre-diabetes	6.5	5	NA

Table 1-1: Circulating leptin in T1D

Some studies report lower or unchanged circulating leptin levels in T1D subjects (bold green), especially in newly diagnosed before insulin therapy. Other reports show higher leptin levels (bold red) either in long duration T1D or after initiating insulin treatment.

3.1.3.2. Influence in T1D

Because of its pro-inflammatory action and its association with IR; leptin may a role in islet autoimmunity in T1D. In support, leptin accelerates T1D in NOD mouse (Matarese *et al.*, 2002). In this study, a leptin surge was observed at a pre-clinical stage before hyperglycaemia but after the beginning of T cell infiltration. Leptin administration at 1µg/kg every 36h for two weeks, favoured early inflammatory infiltration and accelerated diabetes in the female NOD mice. The infiltrating T cells were skewed to a Th1 phenotype due to an increase in IFN-γ production mediated by leptin. In this context, Th1 cytokines favour

initiation and progression of autoimmune responses by influencing antigen processing, presentation and co-stimulation on APCs (Rabinovitch *et al.*, 2003). The leptin effect may then possibly result in either an increase in auto-reactive T cells priming or the reduction of T cell apoptosis.

Although definitive studies are needed in T1D, there is a potential for leptin to play a role in the development of T1D.

3.2. Adiponectin

3.2.1. Adiponectin and its receptors

Adiponectin (AQ) was first identified in mouse adipocytes as adipocyte complement related protein of 30 kDa (Acrp30) (Scherer *et al.*, 1995) and then AdipoQ (Hu *et al.*, 1996). It was then identified in humans by large-scale random sequencing of human adipose tissue cDNA (Maeda *et al.*, 1996) and in plasma as gelatine binding protein (GBP28) (Nakano *et al.*, 1996). Adiponectin is a 28kDa protein secreted by the adipose tissue and encoded by the AdipoQ gene located on chromosome 3q21 (Kissebah *et al.*, 2000). Adiponectin shares structural homologies with the family of the complement C1q (Wong *et al.*, 2004). Its structure includes a carboxy terminal globular head and a collagenous tail. By non-covalent links the collagenous tails associate to form dimers and trimers referred as low molecular weight adiponectin (LMW). Further disulfide bridges and post-transcriptional modifications result in formation of the medium and high molecular weight (MMW and HMW) species present in the circulation along with the LMW form (**Figure 1-9**) (Waki *et al.*, 2003; Tsao *et al.*, 2003; Richards *et al.*, 2006). Adiponectin has also been detected in the blood as a globular form resulting from cleavage of the collagenous tail by leukocyte elastase (Waki *et al.*, 2005).

Two seven transmembrane G-coupled receptors (GPCR) have been identified to specifically bind adiponectin, adiponectin receptor 1 and 2 (AR1 and AR2) (Yamauchi *et al.*,

2003). Both receptors are ubiquitously expressed and differ by their specificity to the different species of adiponectin. AR1 binds preferentially to globular adiponectin while AR2 engage the full-length proteins preferentially (Yamauchi *et al.*, 2003). Both AR1 and AR2 are GPCR integrated in the membrane. However, their N-terminal is intracellular and their C-terminal is extracellular which is the opposite of most GPCR topology (**Figure 1-9**) (Yamauchi *et al.*, 2003).

Sequence alignment reveals that 69 amino acids in the N-terminal of AR1 and 79 amino acids for AR2 are specific for each receptor. Adiponectin binding to both receptors stimulates the activation of AMP-activated protein kinase (AMPK), peroxisome-proliferator activated-receptor- α (PPAR- α), p38 mitogen-activated protein kinase, nuclear factor κ B (NF- κ B) and ERK-1/2 (**Figure 1-9**) (Yamauchi *et al.*, 2003, Tang *et al.*, 2007). Recent studies have identified APPL1 as an intermediate in the coupling of AR1 and AR2 with their signalling pathways (Cheng *et al.*, 2007; Mao *et al.*, 2006). Both AR1 and AR2 have been mainly detected in the adipose tissue and skeletal muscle as well as beta-cells, osteoblasts-like cells and interestingly in PBMC (Pang, Narendran, 2008). In this population, AR1 and AR2 are mainly expressed on monocytes then NK cells and B cells. Little expression is detected on T cells, which express it at high levels intracellularly (Wilk *et al.*, 2011). T-cadherin has also been identified as an adiponectin receptor and is able to activate the AMPK pathway (Hug *et al.*, 2004). It has been recently been shown to mediate the cardioprotective effect of adiponectin in mice (Denzel *et al.*, 2010).

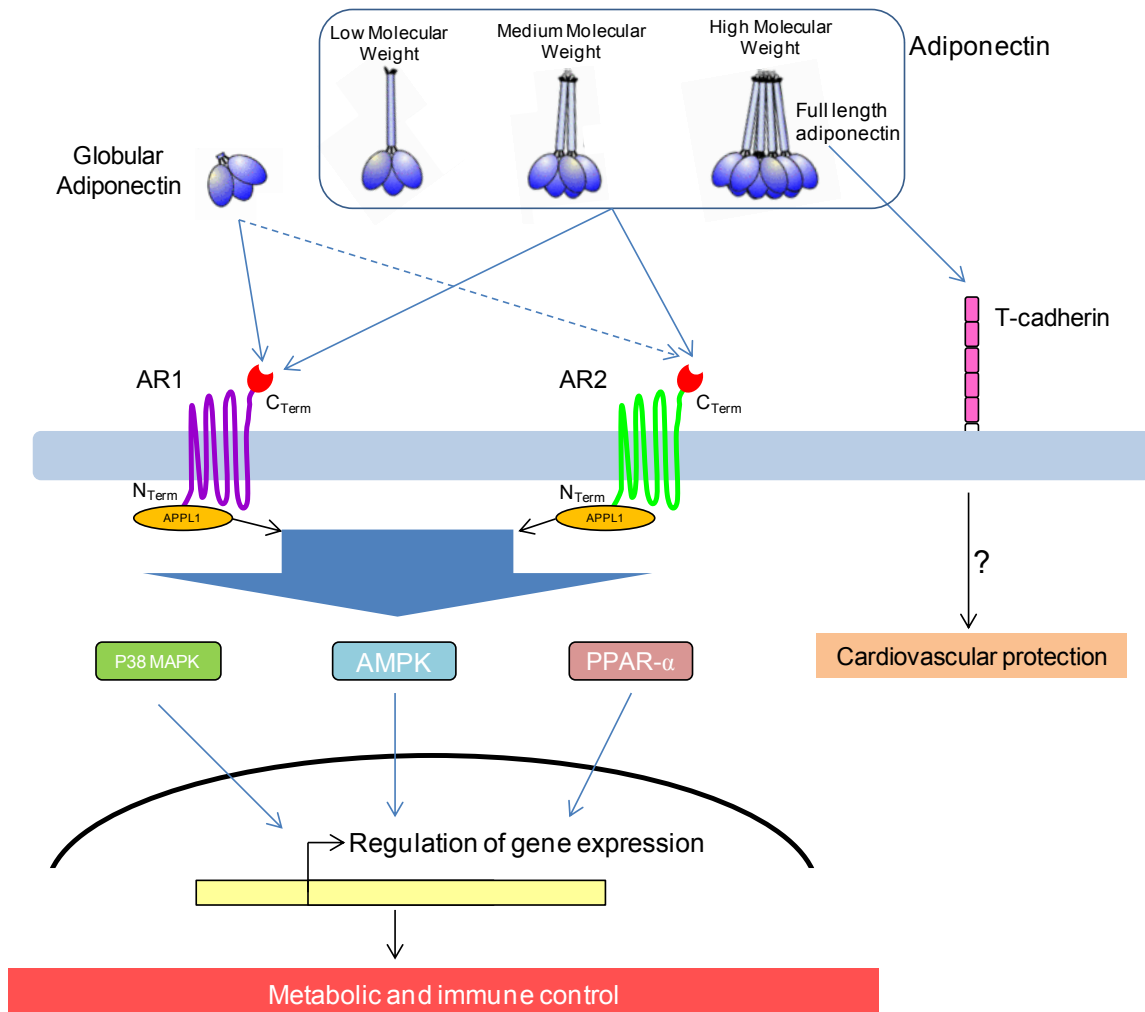


Figure 1-9: Adiponectin signalling pathways

LMW, MMW and HMW preferentially bind to AR2 but can also bind AR1. These receptors can also bind globular adiponectin. AR1/2 are GPCR exposing C-terminal at the cell surface and N-terminal intracellularly. APPL1 is an intermediate for the recruitment of AMPK, PPAR- α or p38 MAPK. The activation of these signalling cascades upon adiponectin binding causes regulation of gene expression that mediates the metabolic and immune control by adiponectin. In addition, T-cadherin is now recognised as another receptor for the HMW of adiponectin and may be involved in mediating its cardiovascular protection role (Adapted from Kadowaki *et al.*, 2008).

3.2.2. Adiponectin role in metabolism

Studies in adiponectin knock-out mouse model revealed a crucial role of adiponectin as an insulin sensitizer (Maeda *et al.*, 2002; Kubota *et al.*, 2002). These mice developed insulin resistance in response to a high fat diet. Replenishment of adiponectin in these mice ameliorates insulin resistance (Yamauchi *et al.*, 2001).

Adiponectin ameliorates insulin sensitivity by stimulating the uptake of glucose and fat oxidation and down-regulating gluconeogenesis and tissue triglyceride contents (Kadowaki *et al.*, 2005). The different forms of adiponectin appear to preferentially induce AMPK, PPAR- α or P38 MAPK pathways depending on the tissue location. For example, globular adiponectin and full-length adiponectin induce AMPK activation in skeletal muscle, but only the full length adiponectin activates AMPK in the liver (Yamauchi *et al.*, 2002). Knocking out these receptors individually has also provided some interesting data. Studies in the murine AR1 knock-out model show that AR1 is responsible for the metabolic effects of adiponectin as these mice are obese with impaired glucose tolerance. Conversely, AR2 knock-out is protective as these mice are resistant to high fat diet-induced obesity (Bjursell *et al.*, 2007).

In humans, adiponectin circulating levels vary between 3 to 30 $\mu\text{g/ml}$, classing it as the most abundant adipokine in the circulation (Arita *et al.*, 1999). In contrast with leptin, adiponectin is reduced in conditions such as obesity and consequently insulin resistance and T2D, as well as coronary heart disease, stroke, non-alcoholic fatty liver disease and several types of cancers (Zhu *et al.*, 2008). It is also recognised that mutations in the AdipoQ gene are associated with T2D (Menzaghi *et al.*, 2007, Li *et al.*, 2009).

3.2.3. *Anti-inflammatory properties of adiponectin*

Adiponectin has been widely reported as an anti-inflammatory cytokine (**Figure 1-10**). It was first evidenced by observation of high levels of the pro-inflammatory cytokines TNF- α and IL-6 in the adipose tissue of adiponectin knock-out mice (Maeda *et al.*, 2002; Uji *et al.*, 2009). Adiponectin anti-inflammatory action has mainly been reported on innate immunity (**Figure 1-10**). Co-culture of adiponectin with macrophages stimulated with lipopolysaccharide (LPS) alters the production of toll-like receptor (TLR)-mediated pro-

inflammatory cytokines such as IFN- γ , IL-6 and IL-8 and their phagocytosis capabilities (Yokota *et al.*, 2000, Wolf *et al.*, 2004). This effect can be mediated by inhibition of the action of NF- κ B (Yamaguchi *et al.*, 2005).

Adiponectin can also induce production of anti-inflammatory cytokines such as IL-10 and IL-1RA, by monocytes, macrophages and DCs (Wolf *et al.*, 2004; Kumada *et al.*, 2004; Wulster-Radcliffe *et al.*, 2004). It has also been reported to alter presentation and co-stimulation by macrophages (Wolf *et al.*, 2004) and foam cell formation. Recent evidence in mice demonstrates a novel effect of adiponectin on DCs function. Indeed, this study shows for the first time that adiponectin reduces differentiation of DCs into efficient APCs as it down-regulates the expression of CD86 as well as up-regulation of PDL-1 (Program death ligand) (Tsang *et al.*, 2010). However, these experiments were conducted in media containing bovine serum that is likely to contain high levels of adiponectin. These DCs were efficient at inducing proliferation of Treg. These observations have been confirmed in humans in our group and using serum-free media. Indeed, monocytes derived DCs express lower levels of CD86 which alter their capacity to prime antigen-specific T cells responses (Pang *et al.*, submitted).

It is becoming clear that adiponectin has an anti-inflammatory action on T cells by modulating APCs, in consistence with the low expression of adiponectin receptors on T cells and very high on APCs. However, direct effects of adiponectin on T cells have also been reported (Wilk *et al.*, 2011). In this study, the authors show that adiponectin receptor expression is up-regulated upon antigen stimulation, resulting in modulation of cytokines profile and induction of apoptosis of antigen-specific T cells. However, these experiments were realised in presence of bovine serum which already contains high levels of biologically active adiponectin. No direct effect has been reported on B cells. The only effect observed for this lineage, is the inhibition of B cell lymphoid progenitor differentiation (Yokota *et al.*,

2003). Adiponectin also inhibits neutrophil and NK cell cytotoxic capacities by inhibition of superoxide and perforin production respectively (Magalang *et al.*, 2006; Kim *et al.*, 2006).

However, controversial data showing pro-inflammatory effects of adiponectin have also been reported. It seems that adiponectin can also induce pro-inflammatory cytokine production such as IL-6, TNF- α and IL-8, by human monocytes and macrophages as well as complement activation and NF- κ B stimulation (Tsatsanis *et al.*, 2005; Peake, Shen, 2010; Haugen *et al.*, 2007).

It has been suggested that the different forms of adiponectin could have different functional effects. For example, low and high molecular weight adiponectin induce apoptosis of monocytes and activation of macrophages suppression capacities in addition to IL-10 production, but the high molecular weight form also induce IL-6 secretion by monocytes (Neumeier *et al.*, 2006). The exact profile of each form of adiponectin is poorly understood and remains to be determined.

These effects could also depend on the experimental settings. For example, difference in duration of LPS stimulation could influence the responses of monocytes and macrophages (Saijo *et al.*, 2005; Tsatsanis *et al.*, 2005). Post-transcriptional modifications observed in mammals might also play an important role in adiponectin-mediated action (Richards *et al.*, 2006), as these are absent from the non-mammalian sources of adiponectin. Finally, it could be attributed to LPS contamination, which is observed in all commercially available sources of adiponectin (Turner *et al.*, 2009).

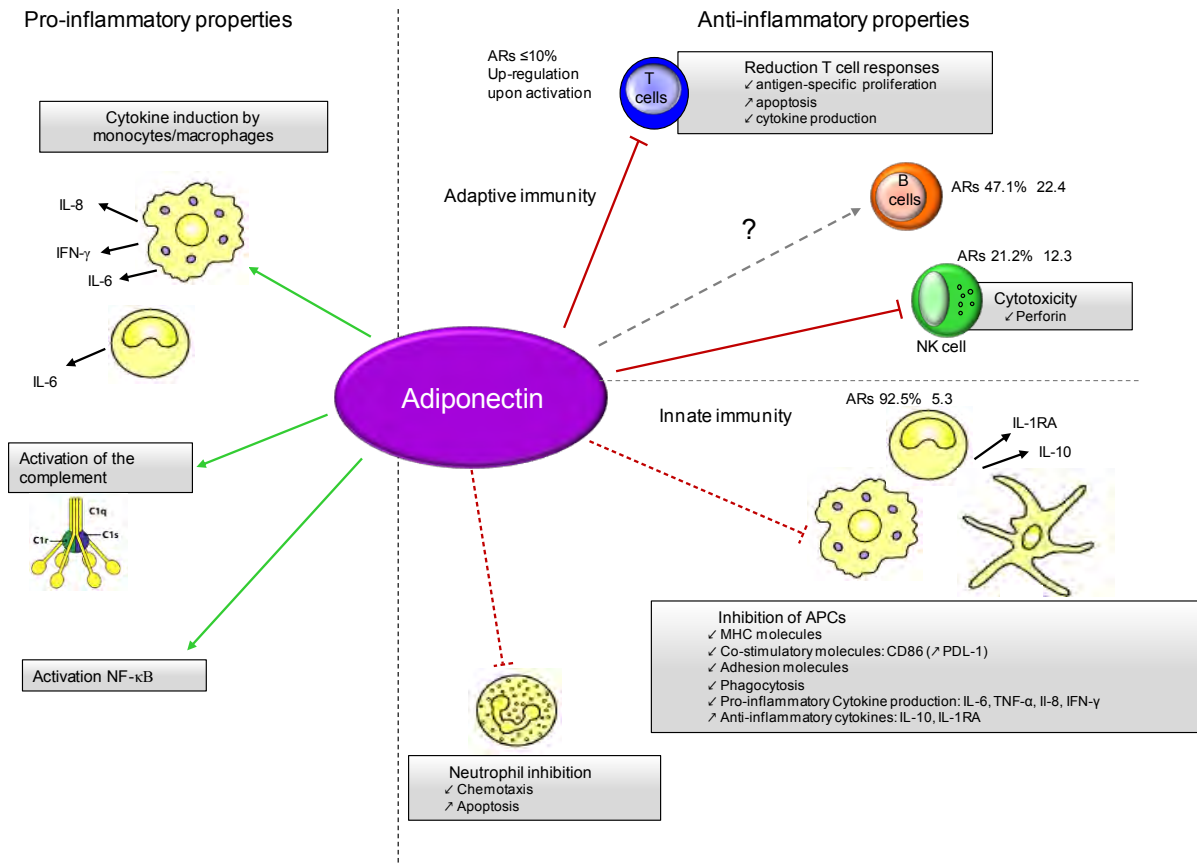


Figure 1-10: Adiponectin and immunity

Several studies have reported the anti-inflammatory action of adiponectin on both innate and adaptive immunity. Adiponectin reduces antigen-specific T cells responses as well as cytokines production and increases rate of T cell apoptosis. Adiponectin can also decrease NK cell cytotoxicity but no effect has been reported on mature B cells. Adiponectin interferes with APCs differentiation by modulating MHC, co-stimulatory and adhesion molecules expression as well as reducing phagocytosis and production of pro-inflammatory cytokines. It can also reduce neutrophil function. On the other hand, pro-inflammatory properties have also been reported for macrophages and monocytes which display higher capacity to produce pro-inflammatory cytokines in presence of adiponectin. In addition, adiponectin is able to activate the complement and NF- κ B pathway. These differences have been attributed to the use of the different form of adiponectin but this issue still need to be addressed.

3.2.4. Adiponectin modulates immune cells migration

Adiponectin concentrations correlate with flow mediated vasodilatation in healthy young men (Torigoe *et al.*, 2007) and low adiponectin levels associate with vascular diseases such as CVD and atherosclerosis in humans (Zhu *et al.*, 2008). Animal models deficient in

adiponectin are more susceptible to vascular disorders (Matsuda *et al.*, 2002). For example, knock-out of adiponectin in the apolipoprotein E (ApoE)-/- mice model of atherosclerosis accelerates the formation of vascular plaque and its over-expression resulted in a marked reduction in atherosclerotic lesions (Okamoto *et al.*, 2002).

Further evidence in the adiponectin knock-out mouse model show that adiponectin attenuates rolling, adhesion and migration of leukocytes through endothelium (Cao *et al.*, 2009, Ouedraogo *et al.*, 2007). In this model, leukocyte rolling and adhesion were increased by 2 and 5 fold respectively. This comes with an increase of VCAM-1 and E-selectin on endothelial cells and a decrease of nitric oxide (NO) production. When adiponectin was administrated to the knock-out mice, these changes were restored. The mechanism underlying the attenuation of transmigration by adiponectin is not fully understood. NO production in endothelial cells helps prevent “over-transmigration” by inducing vasorelaxation and intensifying the blood flow (Huang *et al.*, 2003). Some evidence in animal models show that adiponectin stimulates the production of NO in vascular endothelial cells as well as the phosphorylation of the endothelial NO synthase (eNOS) and this was blocked by inhibition of AMPK (Chen *et al.*, 2003; Motoshima *et al.*, 2004).

In vitro assay treatment of human aortic EC with adiponectin for 18h along with TNF- α , reduces adhesion of THP-1 monocytic cells by inhibition of VCAM-1, E-selectin and ICAM-1 (Ouchi *et al.*, 2000). In addition, over-expression of AR1 in human EC ameliorates adiponectin-mediated suppression of ICAM-1 and NF- κ B expression and activation (Zhang *et al.*, 2009). In this system this is also mediated by stimulation of NO production (Deng *et al.*, 2010).

Other mechanisms involve capture of chemokines by adiponectin. Its complex multimeric nature allows binding of the globular heads to chemokines such as SDF-1, MIP-1 α , RANTES, and MCP-1 (Masaie *et al.*, 2007). This affects chemokine signalling and at the

same time allows adiponectin to be transported to inflamed tissues where it can regulate EC physiology and potentially leukocyte migration.

3.2.5. *Adiponectin in T1D*

In contrast to the low levels observed in diseases such as obesity and T2D, elevated adiponectin concentrations have been reported in chronic inflammatory and autoimmune diseases such as T1D, systemic lupus erythematosus, rheumatoid arthritis and osteoarthritis (Frystyk *et al.*, 2005; Rovin *et al.*, 2005; Senolt *et al.*, 2006; Filkova *et al.*, 2009). Here, adiponectin seem to have contrasting effects. For example, studies have shown a detrimental role for adiponectin as it activates pro-inflammatory processes in joints leading to degradation of the matrix (Ehling *et al.*, 2006). In contrast, adiponectin has been involved in maintenance of cartilage homeostasis in osteoarthritis and anti-inflammatory properties on synovial fibroblast from rheumatoid arthritis patients (Chen *et al.*, 2006; Lee *et al.*, 2008).

Adiponectin is also higher in adults with T1D (Frystyk *et al.*, 2005; Imagawa *et al.*, 2002; Perseghin *et al.*, 2003, Zhi *et al.*, 2011). In addition, high levels of adiponectin were associated with higher all-cause and cardiovascular mortality (Forsblom *et al.*, 2011). Several questions arise from these observations as adiponectin is considered as a protective agent against cardiovascular diseases. There are a number of potential explanations. First, the recruited patients are old and therefore at risk of large vessels disease. The higher adiponectin may therefore be a compensatory mechanism to protect from this complication (Sattar, 2011). They are also likely to have completely lost endogenous insulin secretion. This is responsible for the higher adiponectin levels observed (Cook, Semple, 2010).

Interestingly, circulating adiponectin is decreased in the months before onset of T1D but levels appear to be restored to baseline after onset (Truyen *et al.*, 2007). Lower adiponectin levels before the onset of T1D could therefore contribute to the development of chronic inflammation.

4. Hypothesis and aims

We hypothesise that differences in the leptin and/or adiponectin axis predispose to the activation and/or recruitment of autoreactive T cells to the inflamed pancreatic islets in T1D.

Our overarching aim is to understand how adipokines modulate autoimmunity in T1D.

The specific aims of this thesis are:

1. To characterise the expression and function of leptin and its receptors on PBMC.
2. To examine the effect of T1D on leptin and its receptors.
3. To examine the effect of leptin and adiponectin on T cell migration.

Understanding the mechanism leading to the activation and the infiltration of inflammatory cells into the islets is important because it may help develop strategies to protect insulin-producing beta-cells from the destructive autoimmune process. The potential involvement of leptin and adiponectin is important because therapies such as exercise and other drugs may be used to modulate their circulating levels or receptor expression, and therefore modulate the recruitment of autoreactive immune cells into the islets.

2.CHAPTER 2-MATERIAL AND METHODS

1. Material

1.1. List of main reagents

Reagent	Supplier	Catalogue number	Working concentration/dilution	Use/Application
RNA easy kit	Qiagen	74104	NA	RNA extraction
Random primers	Promega	C1181	NA	Reverse transcription
Superscript II reverse transcriptase	Invitrogen	180-01-002	10U	Reverse transcription
RNase out	Invitrogen	10777-019	10U	Reverse transcription
Superscript buffer	Invitrogen	18064-14	1X	Reverse transcription
dNTPs	Promega	U120-3A	10mM	Reverse transcription, conventional PCR
BioTaq polymerase	Bioline	21040	1U	Conventional PCR
MgCl ₂	Promega	21040	4mM	Conventional PCR
Agarose	Bioline	BIO-41025	5%	Conventional PCR
Loading buffer	Bioline	BIO-37045	1X	Conventional PCR
Ethidium bromide	Fluka	46067	1X	Conventional PCR
Taqman Master Mix	Applied biosystems	P02016	1X	qPCR
Lympholyte-H	VH Bio	CL5010	1X	PBMC isolation
Histopaque 1119	Sigma-Aldrich	11191	1X	PBMC isolation
Histopaque 1077	Sigma-Aldrich	10771	1X	PBMC isolation
PBS	Sigma-Aldrich	P4417	1X	Washes, cell sorting, FACS staining
BSA	Sigma-Aldrich	A7030	5%	Cell Sorting Buffer
EDTA	Sigma-Aldrich	93302	200mM	Cell Sorting Buffer
RPMI 1640	Gibco Invitrogen	21875	1X	PBMC culture
CellGroSCGM	CellGenix	10802	1X	Cell culture
CellGroDC	CellGenix	20801	1X	DCs Differentiation
M199	Gibco Invitrogen	31150	1X	HUVEC culture high serum, PBL assays
Endothelial basal	Promocell	C-22110	1X	HUVEC culture low serum
Optimem	Gibco Invitrogen	51985	1X	Transfection
FCS	Gibco Invitrogen	16000	10-20%	PBMC, HUVEC culture
Penicillin/ Streptomycin	Gibco Invitrogen	10378016	1:500	Cell Culture
Gentamycin	Sigma-Aldrich	G1272	35µg/ml	HUVEC culture
Amphotericin B	Sigma-Aldrich	A2942	2.5µg/ml	HUVEC culture high serum
Epithelial growth factor (EGF)	Sigma-Aldrich	SRP3027	10ng/ml	HUVEC culture high serum
Hydrocortisone	Sigma-Aldrich	H6909	1µg/ml	HUVEC culture high serum
Trypsin	Gibco Invitrogen	25200056	2.5mg/ml	HUVEC culture
PHA	Sigma	L1668	2 to 10µg/ml	T cell activation
CD3/CD28 Beads	Invitrogen	111.31D	1bead to 8 T cells	T cell activation
PMA	Sigma-Aldrich	P1585	50ng/ml	Intracellular Cytokines measurements
Ionomycin	Sigma-Aldrich	I0634	1µM	Intracellular Cytokines measurements
Tetanus Toxoid Protein	Calbiochem	582231	5µg/ml	T cell activation
Proinsulin B-C peptide	Mimotopes	NA	10µg/ml	T cell activation
Tetanus Toxoid Peptide	Mimotopes	NA	10ng/ml	Transmigration
Recombinant Human leptin	R/Dsystems	398-LP	0 to 100nM	Western Blot, functional assays
IL-4	Peprtech	200-04	1µg/ml	DCs Differentiation
GM-CSF	Peprtech	300-03	1µg/ml	DCs Differentiation

Reagent	Supplier	Catalogue number	Working concentration/dilution	Use/Application
LPS	Sigma-Aldrich	L2654	10µg/ml	DCs maturation
CFSE	Invitrogen	V12883	1.25nM	T cell proliferation
Recombinant Human Leptin R/Fc Chimera	R/Dsystems	389-LR/CF	50µg/ml	Anti-human LEPR blocking
Propidium Iodide (PI)	Sigma-Aldrich	P4170	1ng/ml	FACS dead cells staining
FcR Blocker	Miltenyi Biotec	120-000-442	1:20	Fc receptor blocking for FACS specific staining
Permeabilisation buffers	eBioscience	00-5523	1X	Intracellular staining
Dextran-FITC	Sigma-Aldrich	FD-4	0.1mg/ml	DCs uptake
W146	Cayman chemicals	10009109	1µM	Transmigration
S1P	Cayman chemicals	62570	0-100µM	Transmigration
CXCL10	Peprtech	300-12	20ng/ml	Chemotaxis
SDF-1α	Peprtech	300-28A	250ng/ml	Chemotaxis
Prostaglandin D2 (PGD2)	Cayman chemicals	12010	1nM	Chemotaxis
IFN-γ	Peprtech	300-02	10ng/ml	HUVEC activation
TNF-α	R/Dsystems	210-TA-010	100U/ml	HUVEC activation
Adiponectin	NovoNordisk	NA	0 to 15µg/ml	PBL Transmigration
Nucleofector kit T cells	Lonza	VPA-1001	NA	T Cell transfection
Lipofectamine RNAi max	Invitrogen	13778	3µl in 1ml	PBL and HUVEC transfection
Brefeldin A	Sigma-Aldrich	B5936	10µg/ml	Secretion blockade
AMPK compound C	Merck	171261	1µg/ml	Transmigration
Accutase	Sigma-Aldrich	A6964	1X	Phenotyping transmigrated PBL
B cell depletion kit	StemCell	19054	NA	Transmigration
CD4 enrichment kit	StemCell	19052	NA	T cell proliferation
CD19 microbeads	Miltenyi Biotec	130-050-301	NA	Transmigration
CD56 microbeads	Miltenyi Biotec	130-050-401	NA	Transmigration
CD3 microbeads	Miltenyi Biotec	130-050-101	NA	Transmigration
CD25 microbeads	Miltenyi Biotec	130-092-983	NA	Treg depletion
CD14 beads	Miltenyi Biotec	130-050-201	NA	DCs experiments
LS column	Miltenyi Biotec	130-042-401	NA	Cell sorting
Zymosan	Sigma-Aldrich	Z4250	1mg	Mice Peritonitis
Collagenase	Sigma-Aldrich	C5894	1X	HUVEC isolation
Gelatin	Sigma-Aldrich	G1393	2%	HUVEC culture

Table 2-1: List of reagents

1.2. Antibodies

The conjugated antibodies used to detect human markers in flow cytometry are listed in **Table 2-2**. Details of the unconjugated primary anti-human antibodies and their conjugated secondary are presented in **Table 2-3**. Conjugated antibodies against mouse markers are shown in **Table 2-4**.

2. Methods

2.1. Measurement of gene expression

2.1.1. PBMC isolation

Venous blood was collected from healthy volunteers, T1D and T2D subjects following consent. In some cases, anonymised buffy coats were obtained from the Birmingham blood centre. Full ethics approval is in place for this work. PBMC were isolated by density-gradient sedimentation over Ficoll-Hypaque by centrifugation at 800g for 25 minutes using Lympholyte-H (VH Bio). After centrifugation, PBMC were harvested from the Ficoll-Plasma interface and washed twice in fresh RPMI 1640 (Gibco) by centrifugation at 700g for 12 minutes. At the last wash, cells were resuspended in 10ml of RPMI and counted manually using a haemocytometer. Preparations contained both lymphocytes and monocytes populations as determined by flow cytometry based on their forward/side scatter profile.

2.1.2. RNA extraction

Total mRNA was extracted using the RNeasy Minikit (Qiagen) according to the manufacturer's protocol. Briefly, PBMC were first lysed then added to a column, after three washes, mRNA was eluted from the column with water. RNA concentration was measured using Nanodrop spectrofluorimeter (LabTech) and RNA was stored at -80°C.

Antibody	Supplier	Catalogue number	Clone	Optimised volume for 100ul	Use to identified
Human antibodies					
CD3-APC (allophycocyanin)	eBioscience	17-0038-71	UCHT1	5	T cells
CD3-PerCpCy5.5 (Peridinin Chlorophyll Protein Complex-Cyanine 5.5)	eBioscience	45-0037-42	OKT3	5	T cells
CD4-FITC(Fluorescein isothiocyanate)	eBioscience	11-0048-42	OKT4	5	CD4+ T cells
CD4-PE (Phycoerythrin)	BD Pharmingen	555347	RPA-T4	2	CD4+ T cells
CD4-PerCP	BD Pharmingen	345770	SK3	2	CD4+ T cells
CD4-TR (Texas Red)	eBioscience	93-0048	OKT4	7	CD4+ T cells
CD4-APC	BD Pharmingen	555349	RPA-T4	5	CD4+ T cells
CD8-PE	eBioscience	12-0086-73	OKT8	5	CD8+ T cells
CD8-PB (Pacific Blue)	eBioscience	48-0086	OKT8	5	CD8+ T cells
CD45RO-APC	BD Pharmingen	340438	UCHL1	4	Memory T cells
CD25-PE	Miltenyi Biotec	130-091-024	4E3	7	Treg
CD25-APC	Miltenyi Biotec	130-092-858	4E3	7	Treg
FoxP3-APC	Miltenyi Biotec	17-4776-42	PCH101	7	Treg
CD19-PECy7	eBioscience	25-0199	HIB19	7	B cells
CD19-PE	BD Pharmingen	12-0199	HIB19	7	B cells
IgD-PerCpCy5.5	BD Pharmingen	561315	IA6-2	6	B cells subsets
IgM-FITC	Invitrogen	MGM01	NA	6	B cells subsets
CD38-PE	BD Pharmingen	555460	HIT2	6	B cells subsets
CD27-APC	BD Pharmingen	337160	L128	7	B cells subsets
CD56-PE	eBioscience	12-0569-42	MEM188	5	NK cells
CD56-APC	eBioscience	17-0567	CMSSB	5	NK cells
CD14-PE	eBioscience	12-0149-73	61D3	5	Monocytes
CD14-APC	eBioscience	17-0149-42	61D3	5	Monocytes
CD11c-PE	BD Pharmingen	12-0116-101	3.9	5	Monocytes, DCs
HLA-DR-FITC	BD Pharmingen	555811	L243	5	Monocytes, DCs
CD80-FITC	BD Pharmingen	557226	L307.4	5	DCs
CD1a-PE	eBioscience	12-0019	HI149	6	DCs
CD83-APC	BD Pharmingen	334098	HB15E	5	DCs
CD86-FITC	BD Pharmingen	555657	2331	5	DCs
DC-SIGN- PerCpCy5	BD Pharmingen	334098	DCN46	5	DCs
DP-2	R&D Systems	FAB3338F	301108	10	PGD2 receptor
CD49d/CD29-PE ($\alpha_4\beta_1$ /VLA-4)	R&D Systems	FAB17781P	P5D2	3	Integrin
CD18/CD11a-FITC ($\alpha_L\beta_2$ /LFA-1)	R&D Systems	FAB1730F	212701	3	Integrin
CXCR3-PE	US Biological	C8349-02	2Ar1	6	Chemokine receptor
ICAM-APC	BD Pharmingen	559771	HA58	3	Adhesion molecule
IFN- γ -PE	BD Pharmingen	340452	25723.11	0.5	Cytokines
IL-10-APC	BD Pharmingen	554707	JES3-19F1	6	Cytokines
IL-21-APC	BD Pharmingen	560463	3A3 N2.1	3	Cytokines
IL-2-PE	BD Pharmingen	341116	5344.111	0.5	Cytokines

Table 2-2: Anti-human antibodies used in flow cytometry

Antibody	Supplier	Catalogue number/Clone	Working Concentration
Unconjugated primary antibodies			
Mouse Anti-LEPR	R/D Systems	MAB867/52263	10µg/ml
Mouse IgG2b Isotype control	eBioscience	14-4737-85	10µg/ml
Mouse Monoclonal Anti- Leptin	Sigma	L-3160/LEP-13	10µg/ml
Rabbit anti-AR1	Phoenix Peptides	G-001-44	5µg/ml
Rabbit anti-AR2	Phoenix Peptides	G-001-23	5µg/ml
Rabbit anti-S1PR1	Cayman biochemicals	10005228	5µg/ml
Rabbit anti-S1PR4	Cayman biochemicals	13489	5µg/ml
Mouse anti-E-selectin	Sigma-Aldrich	S-9555/1.2B6	1/100
Rabbit anti-VCAM	Abcam	Ab58838-100	1/100
Conjugated secondary antibodies			
Donkey anti-mouse AlexaFluor 488	Invitrogen	A-21202	1/400
Goat anti-rabbit AlexaFluor 488	Invitrogen	A-11008	1/250
Isotypes			
IgG1-APC	Ebioscience	17-4714	0.5-7µl
IgG1-FITC	Ebioscience	11-4614-80	0.5-7µl
IgG1-PE	BD Pharmingen	534680	0.5-7µl
IgG1-PB	Biolegend	400131	0.5-7µl
IgG1-PerCpCy5.5	Biolegend	400531	0.5-7µl

Table 2-3: Unconjugated primary, conjugated secondary antibodies and isotype controls used in flow cytometry

Antibody	Supplier	Catalogue number	Clone	Optimised volume for 100ul	Use to identified
Mouse antibodies					
CD3-FITC	eBioscience	11-0031	145-2C11	1:50	T cells
CD3-PECy7	eBioscience	25-0031	145-2C11	1:50	T cells
CD4-PB	eBioscience	48-041	GK1.5	1:100	CD4+ T cells
CD8-TR	Invitrogen	MCD0817	5H10	1:200	CD8+ T cells
CD11c-PECy7	eBioscience	25-0114	N418	1:50	DCs
CD19-APC	eBioscience	1D3	17-1093	1:50	B cells
CD44-FITC	eBioscience	IM7	11-0441	1:500	Naive/ effector/ memory T cells
CD45-PerCpCy5.5	eBioscience	30-F11	45-0451	1:200	Leukocytes
CD62L-PE	eBioscience	MEL-14	12-0621	1:500	Naive/ effector/ memory T cells
B220-APC Cy7	eBioscience	RA3-6B2	51-0452	1:100	B cells
gp38-PE	eBioscience	8.1.1	12-5381	1:200	Macrophages
F4/80-APC	AbD Serotec	CI:A3-1	MCA497APCT	1:20	Macrophages

Table 2-4: Anti-mouse antibodies used in flow cytometry

2.1.3. Reverse transcription

To convert RNA to cDNA, random primers (Promega) were annealed to 1µg of mRNA for five minutes at 70°C, after which the following mastermix was added to give a final volume of 30µl: 10U Superscript II Reverse Transcriptase (RT), 10U RNAout RNase inhibitor, 1X Superscript Buffer (all from Invitrogen) and 10mM dNTPs (Promega). The reaction was run at 37°C for one hour, followed by five minutes at 95°C. Reactions run without RT were used as negative controls.

2.1.4. Conventional PCR

cDNA (2µl) was amplified using Taq DNA polymerase (1U)(Promega), MgCl₂(4mM), dNTPs (2mM), the appropriate primers at 100µM (**Table 2-5**) and water to complete to 20µl. The reaction was began at 95°C for 5 minutes, then a denaturation step (1) at 94°C for 1 minute, followed by primer annealing (2) during one minute, at the appropriate temperature depending on the pairs used (62 to 70°C) and a elongation step (3) at 72°C for one minute. Step 1, 2 and 3 were repeated for 35 cycles and followed by two minutes at 72°C and 4°C at the end of the reaction. The PCR products were then ran on a 5% agarose electrophoresis gel containing ethidium bromide.

Target	Forward (5'-3')	Reverse (5'-3')	Predicted product size (bp)
For conventional PCR			
Leptin	CAGCGTTTGCAAGGCCCAAGAAGCCC	GAGGAGACTGACTGCGTGTG	300
/LEPR	GAAGATGTTCCGAACCCCAAGAATTG	CTAGAGAAGCACTTGGTGACTGAAC	427
sLEPR	GGGAAGTTGGGCACATTGGGTTCA	CCATTGAGAAGTACCAGTTCAGTCTTACC	329
AR1	TGCCCTCCTTTCGGGCTTGC	GCCTTGACAAAGCCCTCAGCGATAG	526
AR2	GGAGCCATTCTCTGCCTTTC	ACCAGATGTCACATTGCCA	467
For real-time PCR			
sLEPR	Forward :CACACCAAAGAATGAAAAAGC Reverse:AGAATGTCCGTTCTCTTCTGA	Probe: CCCCAAGAATTGTTCTCTGGGCA	140

Table 2-5: Probes and primers used for conventional and real-time PCR

2.1.5. *Real-Time PCR*

Quantitative PCR (qPCR) was performed in duplicate and triplicate on a real-time PCR machine (Stratagene) or ABI 7500 (Applied Biosystems). Each reaction was composed of the adequate quantities of both reverse and forward primers, Taqman probes, Taqman Universal Mastermix (Applied Biosystems), endogenous controls: hypoxanthine phosphoribosyltransferase 1 (HPRT1) or 18S, cDNA and nuclease free water at a total volume of 25 µl. For leptin and its long form receptor (*lLEPR*), Taqman gene expression assay kit (Hs00174492_m1 for *lLEPR* and Hs00174877-m1 for leptin) designed by the manufacturer (ABI) were used, and for the short leptin receptor isoform (*sLEPR*), custom primers and probe were designed (Eurogentec) (**Table 2-5**). For both receptors, primers and probes were picked to target the intracellular region on the receptors enabling to differentiate the different isoforms. Relative quantification of leptin and *sLEPR* and *lLEPR* gene expression, normalised to HPRT1 ran in duplex and was calculated by the $\Delta\Delta C_t$ method, after validating their relative efficiencies on a standard curve. Samples were considered valid when ΔC_t SD > 0.3 (see **Chapter 3**). Expressions of AR1 (Hs00360422_m1), AR2 (Hs00226105_M1), ICAM-1 (Hs0016932_m1), VCAM-1 (Hs00365486_m1), E-selectin (Hs00174057_m1), CXCL9 (Hs00171065_m1), CXCL10 (Hs00171042_m1), CXCL11 (Hs00171138_m1), SPHK1 (Hs01116530_g1), SPHK2 (Hs00219999_m1), SIPR1 (Hs00173499_m1), S1PR4 (Hs02330084_s1) were realised in singleplex reactions on the ABI 7500. Relative expression was calculated by the $\Delta\Delta C_t$ normalised to 18S. All the probes used were labelled with a FAM (tetrachlorofluorescein) marker except for the endogenous controls that were labelled with a VIC marker. Relative expression units (REU) were calculated using the following equation: $REU = 2^{-\Delta C_t}$.

2.2. Measurement of protein expression

2.2.1. Flow cytometry

About 10^6 PBMC were centrifuged at 700g for 7 minutes in polypropylene tubes (BD Bioscience), resuspended by gentle agitation and pre-incubated 2 minutes with 3 μ l of FcR Blocker (Miltenyi Biotec). For immunophenotyping, PBMC were stained for 20 minutes at 4°C with antibodies for PBMC markers (Ebioscience, BD Pharmingen, and Miltenyi Biotec) used at the manufacturer's recommended concentration (**Table 2-2**).

Where directly conjugated antibodies were not available, the following primary and secondary antibody combinations were used to analyse expression of LEPR, leptin, AR1/2, S1P1/4, E-selectin and VCAM-1 as follows (**Table 2-3**). PBMC were stained with the relevant unconjugated monoclonal antibodies during 20 minutes at 4°C. Subsequently samples were labelled with the relevant secondary antibodies conjugated to Alexa 488 (same channel as FITC on the flow cytometer) for 20 minutes at 4°C. Isotype controls and secondary only conditions were used as negative controls.

For intracellular staining, the Ebioscience permeabilisation/fixation kit was used following the manufacturer's protocol. Briefly, PBMC were first permeabilised and fixed in 1:4 ratio of permeabilisation/fixation solution 1 and 2 during 30 minutes at 4°C. The cells were then washed in the permeabilisation buffer at 700g for 10 minutes. 10 μ l of FcR Blocker were then added to the cells suspension and PBMC were stained with the relevant antibodies for 30 minutes at 4°C.

Samples were assayed using a FACSCalibur (BD Pharmingen) with Cell quest software or a Cyan (Dako) with Summix software and then analysed using FlowJo software. Viable cells were routinely gated using Propidium Iodide (2 μ /tube) (PI) and using side-forward scattering. Between 10000 and 50000 events per sample were recorded and plotted as frequency of positive cells or mean fluorescence intensity (MFI).

2.2.2. Western Blot

PBMC cell lysates were obtained in a lysis buffer containing 1% Triton X, 50mM Tris HCl pH8.0, 150mM NaCl and protease inhibitor cocktail (Sigma). Total cellular proteins were analysed using 12% SDS-PAGE under reducing conditions as previously reported. After electrophoresis, proteins were transferred onto a nitrocellulose filter membrane (Amersham) using the I-Blot semi-dry transfer system (Invitrogen). Membranes were blocked with 8% non-fat milk in PBS 0.5% Tween for one hour at room temperature and then incubated overnight at 4°C with the primary antibody at 1/500 to 1/1000 dilutions. Membranes were subsequently washed 3 times in PBS 0.5 % Tween, incubated one hour at room temperature with a secondary goat anti-mouse or goat anti-rabbit IgG antibody conjugated to horseradish peroxidase (Dako) and washed again 3 times in PBS 0.5 % Tween. Peroxidase chemifluorescent activity was detected with the ECL Plus system (Amersham). To confirm the specificity of the bands detected, samples stained with secondary antibody alone or recombinant proteins to block the antibodies during 12 hours before probing were performed (Burnette, 1981).

2.3. ELISA assay

Serum leptin levels were measured in healthy, T1D and T2D subjects using leptin Quantikine ELISA kit (R/D Systems) following the manufacturer's protocol. Serum samples were diluted 1:20 in buffer and plates were read using the Victor Multilabel 1420 counter machine (Wallac-PerkinElmer). The leptin concentrations in the serum were determined using the calibration curve determined using recombinant leptin and were expressed as ng/ml.

2.4. Cell sorting

2.4.1. Positive selection of PBMC subsets

Sub-populations of the following mononuclear leukocytes were obtained by magnetic cell sorting out of pre-isolated PBMC from whole blood following the manufacturer protocol: monocytes (CD14), CD3⁺T cells, B lymphocytes (CD19), and NK lymphocytes (CD56). Briefly, whole PBMC were pelleted at 700g for 10 minutes, labelled with the appropriate anti-CD microbeads and incubated 15 minutes at 4°C. Cells were then washed in PBS 0.5% BSA 2mM EDTA (MACS buffer) and loaded on the prepared LS column (magnetic column) (Miltenyi Biotec) placed in the magnetic field. Unlabelled cells that pass through the column were collected and column was washed three times with 3ml of MACS buffer. The column was then removed from the magnetic field and magnetically labelled cells were flushed out the column by adding 5ml of buffer and flush out a second time with 2ml of buffer. Samples were then counted and labelled with the appropriate anti CDs. Purity and yield was checked by flow cytometry. Purity between 80%-98% was obtained for each sorting and was considered satisfactory.

2.4.2. Negative selection of PBMC subsets

In some conditions, it is essential to use negatively selected or “untouched” PBMC as for instance anti-CD3 microbeads activates T cells and B cells seem to be sensitive to CD19 microbeads.

CD4⁺ CD25⁻ T cells and CD19⁺ B cells were sorted by depletion using the EasySep enrichment kits according to the manufacturer’s instructions (Stemcell). Briefly, the antibody cocktail was added at 50 µl for 5.10⁷ cells in a 15ml falcon tube for 10 minutes at room temperature. The dextran-coupled beads were added at 75µl per 5.10⁷ cells for 5 minutes at room temperature. Total volume was adjusted to 9ml with MACS buffer and the tube was

places in the magnet for 5 minutes. Purified cells were then poured into a new tube and these last two steps were repeated twice to increase purity. CD4⁺ CD25⁻ T cells were then isolated by CD25⁺ depletion using anti-CD25 microbeads. Cell sorting purity was assessed by flow cytometry. Purity between higher than 96% was obtained for each sorting and was considered satisfactory.

2.5. DCs Phenotyping and function

2.5.1. Monocyte-derived DCs generation

Monocytes were isolated by magnetic cell sorting in sterile conditions (described section 2.4.1) and resuspended in serum or serum-free media (CellGroDC supplemented with PS and Glutamine). CD14⁺ monocytes ($5 \cdot 10^5$) were suspended in 500 μ l of media in 24-wells plate. IL-4 and recombinant human granulocyte macrophage colony stimulating factor (GM-CSF) were added to the wells at a final concentration of 1000U/ml each. For some conditions, leptin was added to the DCs at 10nM. Cultures were incubated at 37°C for a total period of 5/6 days and were re-fed at day 2 with either media with serum or CellGroDC containing IL-4 and GM-CSF at 1000U/ml.

Isolated monocytes as well as DCs at day 5/6 were labelled with antibodies against CD14, CD1a, CD80, CD83, CD86, DC-SIGN and HLA-DR and analysed by flow cytometry. Immature DCs (imDCs) were defined as cells with the following expression profile: CD14⁻, DC-SIGN⁺⁺⁺, CD1a⁺⁺⁺, HLA-DR⁺⁺, CD86⁺, CD80⁺ and CD83⁺.

2.5.2. imDC dextran-FITC uptake

imDCs were generated as previously described. 250000-500000 imDCs were transferred into cold (4°C) or 37°C tubes, washed in cold or warm PBS and finally resuspended in 1ml of cold or warm PBS. Dextran-FITC (Sigma) was added at 0.1mg/ml during 10 minutes at 4°C or 37°C. Cells were then washed two times at 700g during 10

minutes at 4°C in cold PBS. Finally, cells were transferred to polypropylene tubes and analysed by flow cytometry. Dextran-FITC uptake was calculated by subtracting the mean fluorescence intensity (MFI) at 4°C to the MFI at 37°C.

2.6. T cell responses measurement

2.6.1. CFSE labelling

Whole PBMC or CD4⁺CD25⁻ sorted T cells were labelled with CFSE in order to follow their proliferation in response to the different stimuli, such as PHA (phytohemagglutinin), CD3/CD28 beads, anti-CD3 and Tetanus Toxoid protein. Cells were washed twice in PBS to remove all traces of foetal calf serum (FCS), counted and resuspended at 2×10^7 cells/ml in PBS. CFSE was added to the cells at a ratio of 1:1 at a concentration of 2.5 μM, vortex and incubated for 10 minutes under agitation at room temperature. After 10 minutes, an equal volume of FCS was added to the solution to quench the labelling reaction and incubated 1 minute. Cells were then washed twice in RPMI 1640 and resuspended to the desired concentration in the appropriate media.

2.6.2. Cell stimulation

PBMC or CD4⁺CD25⁻ T cells and monocytes were cultured in serum (RPMI 1640 supplemented with Penicillin/Streptomycin (PS) and 10% FCS and serum-free conditions (CellGroSCGM supplemented with PS).

To assess T cell responses, T cell proliferation was followed on whole PBMC or fresh sorted CD4⁺CD25⁻ T cells using either monocytes or imDCs as APCs or CD3/CD28 beads as stimulator.

For basic PBMC or CD4⁺CD25⁻ T cells with monocytes stimulation, 2×10^5 CFSE-labelled PBMC or CD4⁺CD25⁻ T cells were suspended in 100 μl of culture media in a 96-wells plate. Cells were stimulated for either 3 days in the presence of 3-5 μg/ml PHA, CD3/CD28

beads (8:1 ratio) or 0.5µg/ml anti-CD3 antibody OKT3 (gift from Prof David Samson, University of Birmingham) made up in 50µl. $2 \cdot 10^4$ monocytes were added to CD4⁺CD25⁻ T cells to provide co-stimulation.

For stimulation of CD4⁺CD25⁻ T cells with imDCs. In order to provide an allogenic stimulation, after sorting CD4⁺CD25⁻ T cells were transferred in a flask in serum media, incubated for 5/6 days during the DCs generation. At day 5/6, CFSE labelling was performed on the CD4⁺CD25⁻ T cells, before co-culture with the imDCs. T cells were plated at a concentration of $2 \cdot 10^5$ cells per 100µl in a 96-round bottom well plate either in serum or serum-free condition with $2 \cdot 10^4$ imDCs and 0.5µg/ml of anti-CD3 during 3-4 days.

For antigen-specific response measurement, 5µg/ml of Tetanus Toxoid protein from *Clostridium tetani* (Calbiochem) was added to the assays and incubated for 7 days.

Proliferation was analysed by flow cytometry by measurement of CFSE dilution.

2.6.3. Intracellular cytokine staining

CD4⁺CD25⁻ T cells stimulated with CD3/CD28 Dynabeads for 5 days were re-stimulated with 50ng/ml of PMA and 1µM of ionomycin for 6 hours. Protein trafficking and secretion was then stopped by addition of 10µg/ml of Brefeldin A for 4-5 hours. Cells were then transferred to a FACS tube and fixed 10 minutes at room temperature in 3% PFA in PBS. Cells were then centrifuged 5 minutes at 700g, permeabilised with 50µl of 0.1% Saponin and centrifuged 5 minutes at 700g. Goat serum was added to block unspecific staining prior to antibodies labelling. Cells were washed two times and analysed by flow cytometry.

2.7. Adhesion and migration assays

2.7.1. Human Umbilical Vascular Endothelial Cells (HUVECs) culture

Human umbilical cords were obtained from the Women's Hospital, Birmingham. Full ethics approval is in place for this work and written consent was obtained before sample was

taken. Endothelial cells were isolated using collagenase digestion as previously described (Cooke *et al.*, 1993). Briefly, cords sections of about 3-4 inches, without clamp or needle damages were sprayed with ethanol 70%. The vein was cannulated and washed twice with PBS, incubated with collagenase (1X) and incubated at 37°C for 15 minutes. Cords were then massaged for one minute and flushed with PBS into a new tube. Cells were centrifuged at 400g for five minutes and resuspended in 5 ml of complete M199 medium (Gentamycine sulphate, 35µg/ml; human epidermal growth factor, 10ng/ml; hydrocortisone, 1µg/ml; penicillin-streptomycin, 1/500; amphotericin, 2.5µg/ml; gentamycin, 35µg/ml and 20% heat inactivated FCS). HUVECs were cultured in 4ml of M199 complete media in 25cm² flasks pre-coated with 2% gelatine.

Confluent HUVECs were washed with a 0.02% EDTA solution and trypsinised. One flask of HUVECs was resuspended in 8ml of low serum endothelial media and seeded in 8 wells of a 12-wells plate. 24 hours later, medium was replaced with low serum endothelial medium containing 10ng/ml IFN-γ and 100U/ml TNF-α for 24 hours.

For the flow conditions, HUVEC were seeded in Ibidi slides (Ibidi). After 24 hours, classic media was replaced by low serum medium containing 10ng/ml IFN-γ and 100U/ml TNF-α for 24 hours.

HUVEC were treated with either 10nM of leptin or 0.0001 to 10µg/ml of adiponectin for 24 hours prior to migration along with the TNF-α/IFN-γ treatment. Due to the AMPK inhibitor Compound C (AMPKi) toxicity overnight, TNF-α/IFN-γ treated HUVEC were incubated 30 minutes with 1µg/ml of AMPK inhibitor. The compound was then washed and adiponectin was added for one hour and finally washed prior to transmigration assay.

HUVEC were also purchased from Provitro, passaged three times in low serum endothelial media (Promocell) and frozen in low serum CryoSFM freezing media (Promocell). Four days before the transmigration experiment, HUVEC were defrosted and

cultured into 25cm² flasks. After 2 days, cells were transferred into 12 well plates and used in transmigration assay.

2.7.2. PBL Migration assay through HUVECs endothelium

2.7.2.1. Static assays

PBMC were isolated from blood by histopaque gradient. Lymphocytes were prepared by panning PBMC onto culture plastic during 30 minutes at 37°C to remove the monocytes (Rainger *et al.*, 2001). Peripheral Blood Lymphocytes (PBL) were then counted, resuspended in M199 2% BSA at 1million cells/ml. Prior to transmigration, 1.10⁶ PBL were treated with adiponectin at 0.0001 to 15µg/ml at room temperature under agitation and washed after one hour.

For the AMPKi experiments, 1.10⁶ cells were incubated 30 minutes at room temperature under agitation with AMPKi compound C at 10µg/ml. Cells were then washed and incubated one hour with adiponectin as described above.

To obtain the following PBL subsets listed in **Table 2-6**, we used negative or positive selection as described in section 2.4:

Before addition of lymphocytes, HUVEC were washed twice with M199 0.15% BSA to remove the excess cytokines. 1x10⁶ of PBL or different fractions listed above, were added to the HUVEC for 6 minutes at 37°C.

Non-adherent cells were removed by gentle wash with M199 0.15% BSA. Each well was then video recorded using a phase-contrast videomicroscope. To measure the percentage of migration, five fields were briefly and randomly recorded. Velocity of cells underneath the

endothelium was recorded during 5 minutes on a single field. Videomicroscopy was realised in a Perspex box allowing maintenance of temperature at 37°C (**Figure 2-1a**).

Fraction	Definition	Mode of selection	Concentration , ratio	Treatments
T cells	CD3 ⁺ T cells	Positive selection of CD3 ⁺ B cells	1x10 ⁶	Adiponectin- 1h
Bs-ve PBL	B cell negative PBL	Positive selection of CD19 ⁺ B cells	1x10 ⁶	Adiponectin- 1h S1P-30min
Bs reconstitution	B cell negative PBL + B cells added back	Negative selection of B cells added back	10:1 (Bs-vePBL: B cells added back)	Adiponectin on B cells added back-1h Pre-treatment of Bs-ve PBL with W146 (10µM) for 30min
NKs	NK cells	Positive selection of CD56 ⁺ NK cells	1x10 ⁶	Adiponectin- 1h
Bs + NKs	NK cells + B cells added back	Bs:Negative selection of B cells added back; NKs: Positive selection of CD56 ⁺ NK cells	10:1 (NKs: B cells added back)	Adiponectin on B cells added back-1h
NKs + Ts	NK cells + T cells	Ts: Positive selection of B cells and NK cells; NKs: Positive selection of CD56 ⁺ NK cells	10:1 (Ts: NK cells added back)	Adiponectin on NK cells added back-1h
Sup Bs PBL	Supernatant B cells on Bs-ve PBL	Negative selection of B cells	1x10 ⁶	Adiponectin on B cells, centrifugation and supernatant added on Bs-ve PBL for 30 min
BrefA Bs PBL	B cell negative PBL + B cells treated with Brefeldin A added back	Negative selection of B cells added back	10:1 (Bs-ve PBL: B cells added back)	Brefeldin A-4h and adiponectin in the last hour

Table 2-6: List of PBL subsets and treatments used in adhesion and migration assays

2.7.2.2. Flow assays

The Ibibi slide was connected to a Harvard withdrawal syringe pump, which delivered flow at a rate equivalent to a wall shear stress of 0.1 Pa. At the other end, the channel was connected to an electronic switching valve (Lee Products, Gerards Cross, UK), which selected flow from two reservoirs containing PBL in PBS 0.02%BSA (PBSA) or cell-free PBSA (**Figure 2-1a, c**). PBL were perfused for 4 minutes over the HUVEC followed by cell-free wash buffer during approximately 2 minutes. Video recordings were made of a series of microscope fields along the centreline of the flow channel after the 2 min of washout.

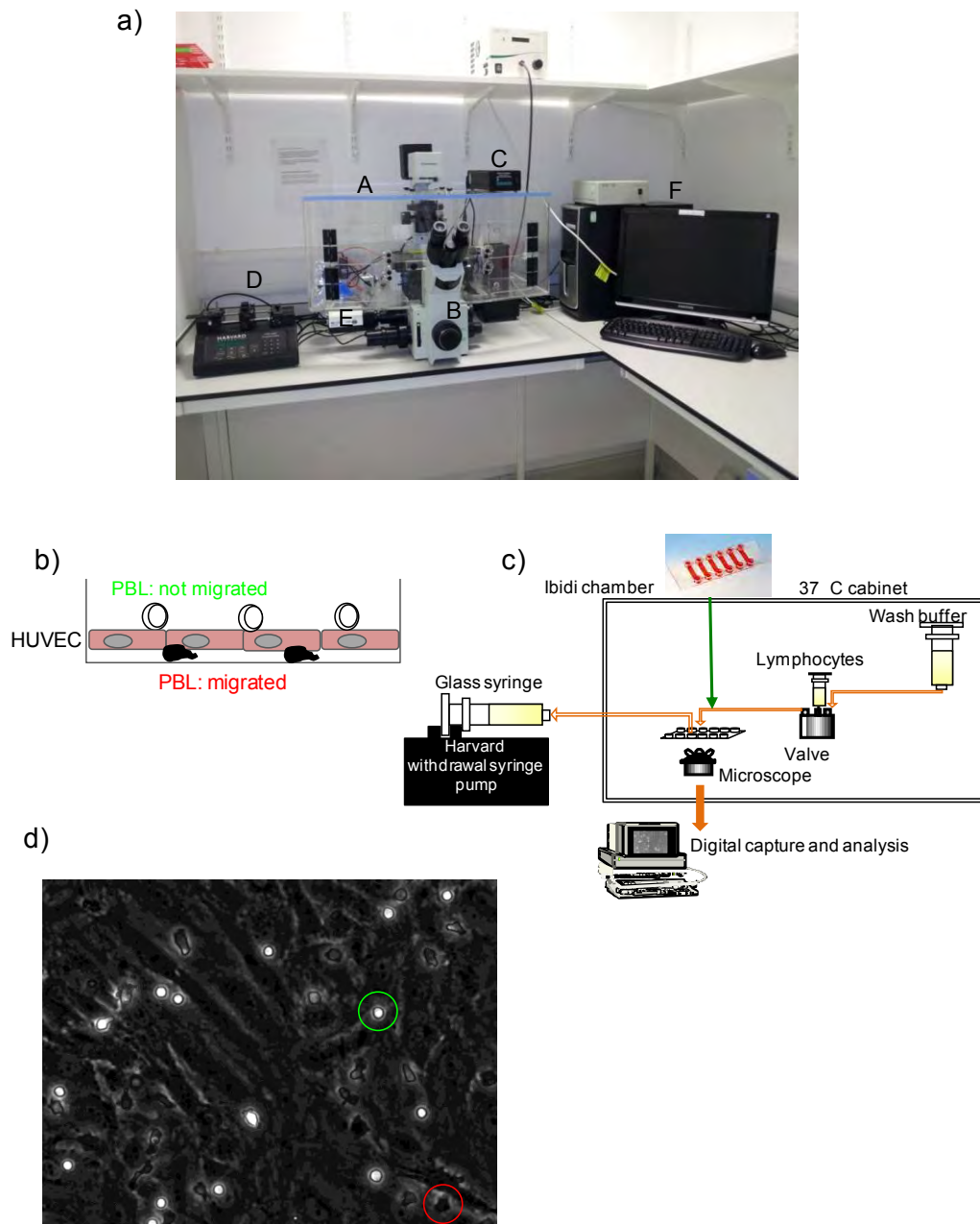


Figure 2-1: Illustrations of the static and flow systems

(a) Photographic representation of the system used to record PBL adhesion and migration. A: Perspex box, B: Phase contrast microscope, C: Heating block, D: Harvard withdrawal syringe pump, E: Video camera, F: computer. (b) In both static and flow assays, phase bright PBL (white circles) are firmly adhered on the top of the endothelium and phase dark PBL (black spread cells) have transmigrated across the endothelium. (c) Schematic representation of the flow system. (d) Example of a field recorded with a phase bright PBL circled in green and a phase dark PBL circled in red.

2.7.2.3. *Analysis of the video recordings*

The recordings were analysed with Image-Pro 6.2 software (Media Cybernetics). In both static and flow conditions, phase bright lymphocytes on the HUVEC surface were classified as adherent (typically with distorted shape and actually migrating slowly on the surface). Phase dark lymphocytes were identified as transmigrated cells (spread and migrating below the surface of the HUVEC) (**Figure 2-1b, d**). Both populations were counted and averaged for the 5 fields and the percentage of transmigrated lymphocytes was calculated. The number of total adherent cells was calculated in cells per mm² using the calibrated microscope field dimensions and multiplied by the known surface area of the HUVEC to calculate the total number of adherent cells. Total adhesion normally ranged between 350 to 680 cells/mm² on stimulated endothelium. Under flow conditions, some phase bright adherent lymphocytes were classified as rolling-adherent (spherical cells moving over the surface much slower than free flowing cells).

2.7.3. *Phenotyping of migrating lymphocytes*

After acquisition, HUVEC monolayers were gently washed with EDTA 0.02% until adherent lymphocytes were removed (top fraction) in both static and flow assays. HUVEC were treated with accutase for 30s at 37°C and the reaction was blocked by adding M199 10% FCS. Both top and migrated lymphocytes fraction were harvested and labelled for flow cytometry.

2.7.4. *Adiponectin receptors, SPHKs knock-down*

2.7.4.1. *On HUVEC*

HUVEC were plated in a 12 well plate (87500 cells per well) for 24 hours or until about 80% confluence. The relevant siRNA (**Table 2-7**) were added at a final concentration of 50nM to 83.75µl or 82.5µl if duplex of Optimem media, 1.5µl of RNAi Lipofectamine was

mixed with 13.5µl of Optimem and these were incubated 10 minutes at room temperature. 15µl of Lipofectamine mix was added to each siRNA singleplex or duplex, gently mixed and incubated for a further 10 minutes. HUVEC were washed twice with PBS and 400µl of Optimem was added to the Lipofectamine siRNA duplexes. After gentle agitation, the mix was added on the HUVEC and incubated at 37°C for four hours. The mix was then replaced with the classic low serum media without antibiotics. After 48 hours, HUVEC were stimulated with TNF- α /IFN- γ for an additional 24 hours before measuring PBL adhesion and migration as described previously. For the SPHKs knock-down experiments, siRNA concentrations were increased to 100nM and transfection was realised twice, once as before, then left for 24 hours and once prior to cytokines stimulation. Scramble siRNAs (Thermo Scientific) pool and untransfected cells were used as negative controls.

ON-TARGETplus SMART pool (Thermo Scientific)				
Target	siRNA1	siRNA2	siRNA3	siRNA4
AR1	GACAAGAGCAGGCGUGUUC	GGCUAAAGGACAACGACUA	CCUUUAUGCUGCUCGAAUU	GAGAAGGGCAAACGGGUAA
AR2	GGAGUGAGGUACAAGACGA	GAGAAGAACUUGAACGUUU	CAGACUUGCUCUUCGGUCA	GAAGGUCGGUGGCGAGUGA
SPHK1	GGAAAGGUGUGUUUGCAGU	GAAAUCCUUCACGCUGA	GAUGGGGAAUUGAUGGUUA	CGACGAGGACUUUGUGCUA
SPHK2	CCACUGCCCUACCCUGUCU	GCUCCUCCAUGGCGAGUUU	GAGACGGGCUGCUCCAUGA	CAAGGCAGCUCUACACUCA

Table 2-7: Sequences of siRNA pool used for HUVEC and lymphocytes

2.7.4.2. On lymphocytes

Transfection of PBL with the relevant siRNA (**Table 2-7**) was performed using the Amaxa technology following the manufacturer's instructions (Lonza). Briefly, 1×10^6 PBL in 100µl of Nucleofactor buffer was supplemented with 100nM of the relevant siRNA, scramble control or siRNA-FTIC (ThermoScientific). The mix was transferred to an electroporation cuvette and programme U-014 was applied on the Amaxa machine. At the end of the program, 500µl of RPMI 10% FCS without antibiotics was added to the cuvette and the mix was transferred to 1.4ml of RPMI 10% FCS into a 6 well plate. PBL were also transfected

using a lipid-mediated delivery. The relevant siRNA (**Table 2-7**) were added at a final concentration of 100nM to 9.5µl or 9µl if duplex of Optimem media, 2µl of RNAi Lipofectamine was mixed with 8µl of Optimem and these were incubated 10 minutes at room temperature. 10µl of Lipofectamine mix was added to each siRNA singleplex or duplex, gently mixed and incubated for a further 10 minutes. After gentle agitation, the mix was added on the PBL, 80µl of RPMI 10%FCS without antibiotics was added and the cells were transferred into a 96 well plate.

For both mode of transfection, PBL were incubated for 48 hours before staining for the relevant targets and analysis by flow cytometry.

2.8. Identification of Agent X

2.8.1. Purification of supernatants

B cells (2×10^5 - 5×10^5) were incubated in presence or absence of adiponectin at 15µg/ml. Adiponectin (15µg/ml) was added in M199 and used as a negative control. The peptides from the three samples were purified using C18 solid phase extraction column from Supelco (DSC-18). The column was conditioned by adding 1ml of 0.1% trifluoroacetic acid (TFA) in acetonitrile (ACN), which like all additions was allowed to drip through the under column under gravity. The column was then equilibrated with 0.1% TFA/water and the sample, adjusted to 0.1% TFA, was added to the column. The column was washed with 1ml of 0.1% TFA/water and the peptides eluted using 0.1% TFA/acetonitrile (1ml) which was dried under vacuum and the samples resuspended 20 µl in 0.1% formic acid in 2% acetonitrile.

2.8.2. Mass spectrometry

2.8.2.1. Principle

The samples were analysed using the (Liquid chromatography) LC-MS/MS (Tandem Mass spectrometry) system described in **Figure 2-2**. The sample was sprayed in the mass spectrometer after the peptides were resolved using a C18 reverse phase nano-bore column using High pressure Liquid Chromatography (HPLC). Briefly, in this approach the samples are loaded onto the column in a low concentration of solvent allowing virtually all the peptides to bind, and the peptides are then eluted using a gradient of increasing solvent such that the more hydrophobic the peptide is the longer it is retained on the column.

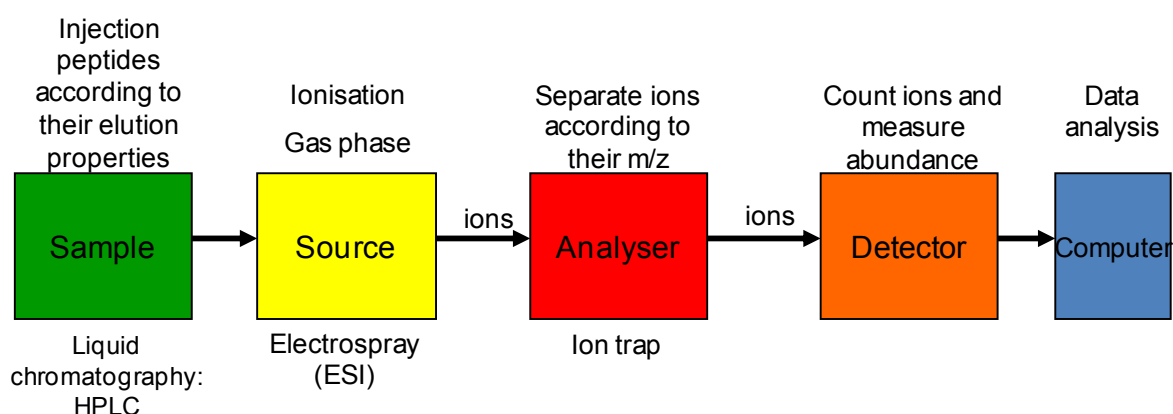


Figure 2-2: Schematic representation of the mass spectrometry system used for the identification of Agent X.

The HPLC eluate is linked directly to an electro spray ionization (ESI) based mass spectrometer which generates gas phase ions in the electrospray source by creating a very fine aerosol (a Taylor cone) as the eluate exits the end of the nano-bore HPLC system, that facilitates evaporation of the eluate liquid. This is aided by heating the source (180°C). By applying a strong electric field (1-2 Kv) to eluate the peptides becoming ionized and are drawn into the mass spectrometer by an electrostatic attraction where they are transferred into

the actual ion-trap using quadrupoles to direct their movement. The ion-trap allows separation of the ions according to their mass to charge ratio (m/z). The ion trap works by trapping ions between five electrodes by applying an electromagnetic field in three dimensions and an initial MS analysis determines the charge to mass ratio (m/z) of the ions in the sample. The ion-trap is then programmed to isolate the most abundant ion species and fragment it by increasing vibrational energy and intruding a small amount of nitrogen such that the ions collide with the inert gas and the break or fragment (collision induced dissociation or CID), typically at the peptide bond. The m/z values of the fragments are then determined which is termed MS/MS. Some newer mass spectrometers can also fragment ions using a different approach, termed electrotransfer dissociation (ETD), which is complementary to CID. LC-MS/MS displays high resolution and sensitivity and allows fragmentation of the selected parent ions giving products ions, essential to determine the sequence of the parent ion and identify the peptide in a protein database.

2.8.2.2. *Experimental design*

10 μ l of the purified samples was subjected to an LC-MS/MS analysis using a gradient of 2-36% ACN in 0.1% formic acid over 30 min at 350nL/min using a Dionex Ultimate 3000 HPLC system. The HPLC column was an Acclaim PepMap100 (75 μ M I.D. x 25cm C18 3 μ M particle size) connected to a Bruker ETD Amazon ion trap mass spectrometer with an online nanospray source fitted with a metal needle with a 10 μ M tip. 1,600 volts was applied to the end plate and the drying gas set to 6.5 litres/min at 180°C. An MS survey scan from 350 to 1600 m/z was performed and the five most intense ions in each survey scan were selected for CID (collision induced dissociation) fragmentation. After ions were fragmented twice they were placed on an exclusion list for 0.5 min. The raw data was processed using the Bruker DataAnalysis peak detection program to select peaks which were then searched using the Mascot search engine (version 2.1) using the SwissProt protein database. The minimum mass

accuracy for both the MS and MS/MS scans were set to 0.5Da and no protease selection was used. The peptides were filtered using a minimum Mascot score of 30. The data output was analysed via the Bruker ProteinScape software package.

2.9. In vivo experiments

2.9.1. Mice

C57BL/6 and BALB/c mice were housed in the animal facilities at the University of Birmingham. BALB/c B cells knock-out (Jh^{-/-}) were generated and kindly donated by Lucy Walker. All experiments were performed in accordance with United Kingdom Home Office regulations. Mice were used between 6 and 8 weeks of age and were sex and age matched unless otherwise indicated.

2.9.2. Peritoneal inflammation

Peritonitis was induced by the intraperitoneal injection (IP) of 1mg type A zymosan as previously described (Rajakariar *et al.*, 2008). Peritoneal cavity washout cells were collected with PBS at 48h post injection. Erythrocytes in the peritoneal exudates were lysed and cells were enumerated with a Counter Coulter before FACS staining (antibodies **Table 2-3**). Tubes containing the cells from the peritoneal exudates were fully acquired on the FACS to accurately count the cells. Gates were set up on all cells in the peritoneum and the number of T cells was determined based on CD3 expression. Blood was drawn by cardiac puncture under the armpit and processed like the peritoneal exudates.

Agent X was administrated to the B cell KO mice as following:

Day 1: Intravenous tail injection of Agent X or scramble (100µg)

Day1 + 4 hours: Intraperitoneal injection of Zymosan (1mg) or PBS + Agent X or scramble (100µg)

Day 1 + 24 hours: Intravenous Tail injection of Agent X or scramble (100µg)

Day 1 + 48h: Cardiac puncture + peritoneal lavage

3. Statistics

Data are expressed as mean \pm standard deviation (SD) or standard error of the mean (SEM). Data have been analysed using Prism software, using one or two-way ANOVA followed by Dunnett's or Bonferroni's multiple comparisons post-test; and t-test for normal Gaussian distributions. If the data did not pass the Kolmogorov-Smirnov normality test, data were analysed using Mann-Whitney t-test. P-value less than 0.05 were considered significant.

3.CHAPTER 3- ANALYSIS OF LEPTIN AND LEPTIN RECEPTOR EXPRESSION IN HEALTH AND DIABETES

1. Introduction

Leptin acts through ObR-a and ObR-b receptors in mice, and through the short and long isoforms of the receptor (*sLEPR* and *lLEPR* respectively) in humans. A number of studies have now reported *sLEPR* and *lLEPR* expression by PBMC. Some studies have analysed expression of both receptors on whole human PBMC using semi-quantitative PCR (Tsiotra *et al.*, 2000). *LEPR* expression had also been reported on CD4⁺ and CD8⁺ T lymphocytes (Martin Romero *et al.*, 2000; Lord *et al.*, 1998 and 2002) as well as monocytes (Zarkesh-Esfahani *et al.*, 2001) and DCs (Mattioli *et al.*, 2005) using flow cytometry and semi-quantitative PCR. *LEPR* expression has also been widely reported on mouse lymphocytes, DCs, NK cells and Treg using semi-quantitative PCR, western blotting, flow cytometry and confocal microscopy (Zhao *et al.*, 2003; Siegmund *et al.*, 2004; Papathanassoglou *et al.*, 2006; De Rosa *et al.*, 2007). Similarly, leptin expression has been reported in mouse effector CD4⁺CD25⁻ T cells, Treg and monocytes using confocal microscopy and immunoblotting (De Rosa *et al.*, 2007; Sanna *et al.*, 2003; Matarese *et al.*, 2005). These observations suggest the existence of a local autocrine loop for the pro-inflammatory action of leptin.

Interestingly, leptin-deficient mice are protected from nephrotoxic nephritis, a form of autoimmune kidney disease (Tarzi *et al.*, 2004) and encephalomyelitis (EAE). A surge in leptin levels is observed in mice before development of EAE (Sanna *et al.*, 2003). Leptin administration to these mice worsens disease by increasing the secretion of pro-inflammatory cytokines (Matarese *et al.*, 2001a and b) and leptin neutralization results in protection from disease (Matarese *et al.*, 2001b). In humans, subjects with multiple sclerosis have elevated plasma leptin (Matarese *et al.*, 2005). This increase correlates with increased circulating pro-inflammatory cytokines as well as reduced Treg number.

With regard to T1D, a study of leptin administration to NOD mice has demonstrated significant acceleration of disease, associated with about three times more histological lesions than the PBS control (Matarese *et al.*, 2002). Furthermore, females NODs (which are more disease prone than male) had higher circulating leptin levels. These data suggest a pro-inflammatory effect of leptin in islet immunity.

To our knowledge, no study has investigated a role of leptin and its receptors in T1D. In this chapter, we aimed to characterise the expression of leptin and both of its receptors on human PBMC in health and T1D.

2. Results

2.1. Leptin receptors are expressed by PBMC.

2.1.1. Gene expression of *sLEPR* and *lLEPR* on PBMC.

We first aimed to confirm the expression of both leptin receptors isoforms at mRNA level using conventional PCR (35 cycles of amplification). We used intron-spanning primers and we showed amplification products at the expected size for *sLEPR* (329pb) and *lLEPR* (427pb) on whole PBMC from healthy controls (HC) and T1D subjects (**Figure 3-1**). mRNA from pancreas and pre-adipocytes were used as positive controls. The reverse transcriptase (RT) negative (-ve) and water controls were negative for each condition, thus revealing that the amplification was specific to mRNA and not to genomic DNA (gDNA) contamination. Expression of *lLEPR* was found on CD3⁺ T cells and also on monocyte-derived immature and mature DCs (**Figure 3-1a, b**).

The expression of *lLEPR*, *sLEPR* and leptin was quantified by real-time PCR. First, the technique for relative quantification in duplex was validated using the $\Delta\Delta C_t$ method (Livak, Schmittgen, 2001). Standard curves were measured by dilution series of the target gene against the endogenous control, HPRT1. In singleplex reaction, the C_t of the dilutions

for *l*LEPR, leptin and *s*LEPR were parallel to HPRT1 with an R^2 close to 1 indicating the goodness of the curve fitting (**Figure 3-2a, c, e**). The efficiency of the reaction is also shown by plotting the ΔC_t (C_t target gene- C_t endogenous control). In this case, the slope gradient being ≤ 0.1 shows the reliability of HPRT1 used as an endogenous control with the three targets genes *l*LEPR, *s*LEPR and leptin (**Figure 3-2b, d, f**). Quantitative measurement of both receptors and leptin by real-time PCR showed their expression on whole PBMC from HC and T1D subjects (**Chapter 3-section 2.5**).

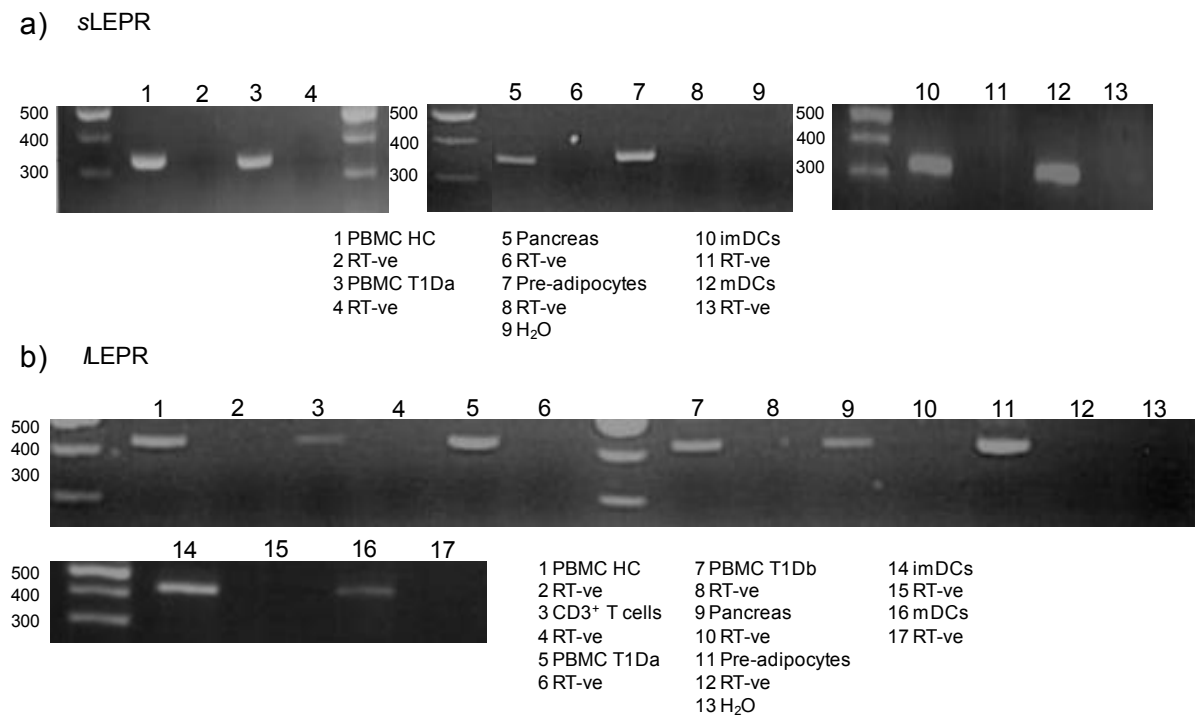


Figure 3-1: LEPRs are expressed on PBMC

(a) *s*LEPR and (b) *l*LEPR expression can be detected in total PBMC mRNA from HC and T1D (patients a and b) by RT-PCR. RT-ve and water controls demonstrate absence of gDNA contamination in the samples. Pancreas and pre-adipocytes mRNA were used as positive controls. The predicted amplicon size for *s*LEPR was 329bp and for *l*LEPR was 427bp. Data are representative of two independent experiments.

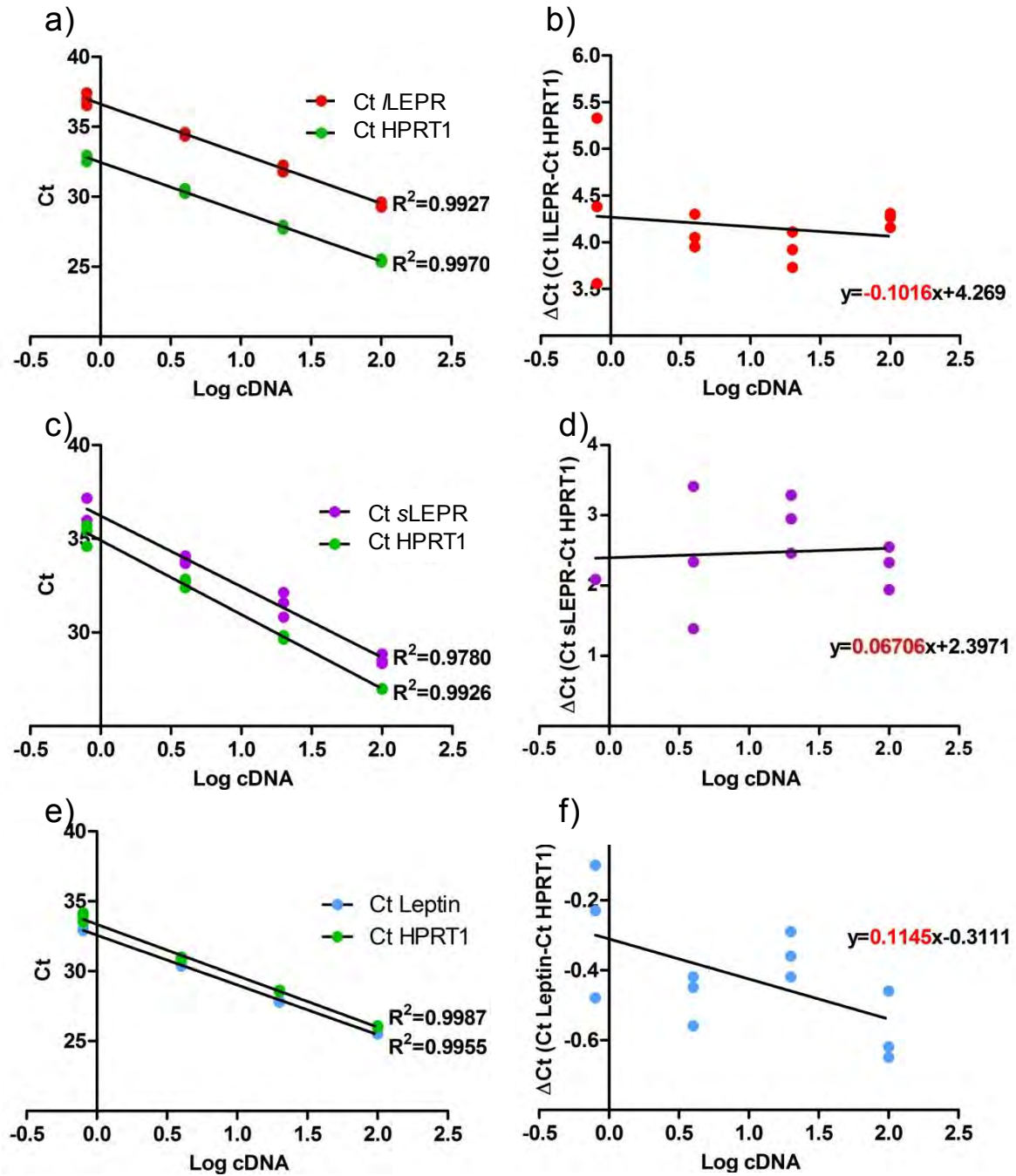


Figure 3-2: Validation of quantitative real-time PCR in duplex

(a, b) Ct and Δ Ct were determined for lLEPR, (c, d) sLEPR and (e, f) leptin after serial dilutions of pre-adipocytes mRNA converted into cDNA. Linear regressions are shown for each condition and the R^2 and the slope (red), determined by the equation $y = ax + b$, were used to measure the amplification efficiency of the target genes in duplexes.

2.1.2. *Distribution of LEPR on PBMC and subpopulations at a protein level*

Then we aimed to quantify LEPR expression on PBMC at a protein level by flow cytometry. Anti-LEPR antibody and its secondary antibody concentrations were first optimised and the optimal concentrations were determined to be 10µg/ml for the anti-LEPR antibody and 5µg/ml for the secondary antibody. LEPR appears to be expressed on whole PBMC, but also on lymphocytes and monocytes subpopulations (**Figure 3-3**). The labelling specificity of anti-LEPR was validated by the absence of staining with the IgG1 isotype control and by specific blocking with a recombinant LEPR protein. PBMC were co-stained with anti-LEPR and phenotypic markers to assess its expression on each CD4⁺, CD8⁺, CD14⁺, CD19⁺ and CD56⁺ cell subtypes (**Figure 3-3 b, c, d, e, f**). The specificity of labelling was shown on each subpopulation with the isotype and the blocking controls. LEPR expression was determined on viable cells recognised by propidium iodide (PI) and forward/side scatter gating (**Figure 3-3a**).

We observed an average LEPR frequency of 6.1±4.3 % for CD3⁺ T cells. Within this population, CD4⁺ and CD8⁺ T cells have 19.7±24.1% and 13.1±12.4% of LEPR positive cells respectively. The proportion of LEPR on CD19⁺ cells and CD14⁺ monocytes is 41.8±21.8% and 82.9±12.7% respectively. Only 7.8±5.7% of CD56⁺ NK cells expressed the surface receptor (**Figure 3-4c**). Treg were identified on basis of CD4⁺CD25⁺FoxP3⁺ expression (**Figure 3-4a**). FoxP3 expression was detected only on the CD4⁺CD25⁺ population as shown by the red histogram and about 20.5±11.8% of Treg expresses LEPR. LEPR expression has also been detected on monocyte-derived DCs (27±5.1%) (**Figure 3-4b, c**). All together, we observed that monocytes are the main cells expressing LEPR, followed by DCs, B cells and Treg. T lymphocytes also express the receptor but at a lesser extent. A similar profile was observed when considering mean fluorescence intensities on each subset (**Figure 3-4d**).

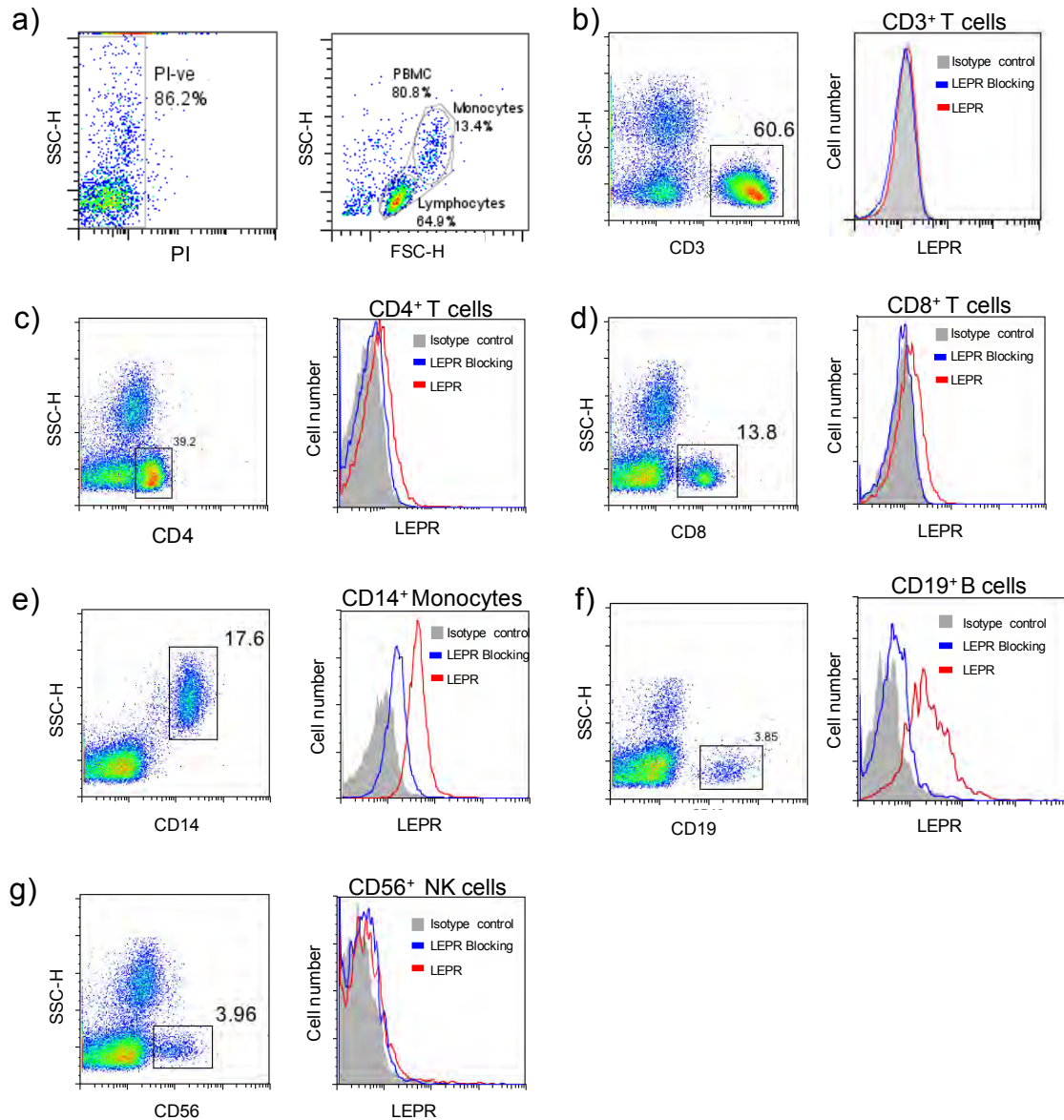


Figure 3-3: Distribution of LEPR expression on PBMC subpopulations

(a) Cells were co-labelled with the different cell markers and anti-LEPR antibody and analysed by flow cytometry. The upper row shows gating on viable cells based on PI and forward/side scatter. Monocytes and lymphocytes populations can be distinguished on the forward/side scatterplots. The other dot plots show gating on PBMC subpopulations (b) CD3⁺ T lymphocytes, (c) CD3⁺CD4⁺ T lymphocytes, (d) CD3⁺CD8⁺ T lymphocytes, (e) CD14⁺ monocytes, (f) CD19⁺ B lymphocytes and (g) CD56⁺ NK cells where LEPR expression was measured as shown on histograms (red). Isotype control (solid grey) and blocking (blue) of the LEPR staining show the specificity of the LEPR labelling for each subset of PBMC. Data are representative of at least three independent experiments.

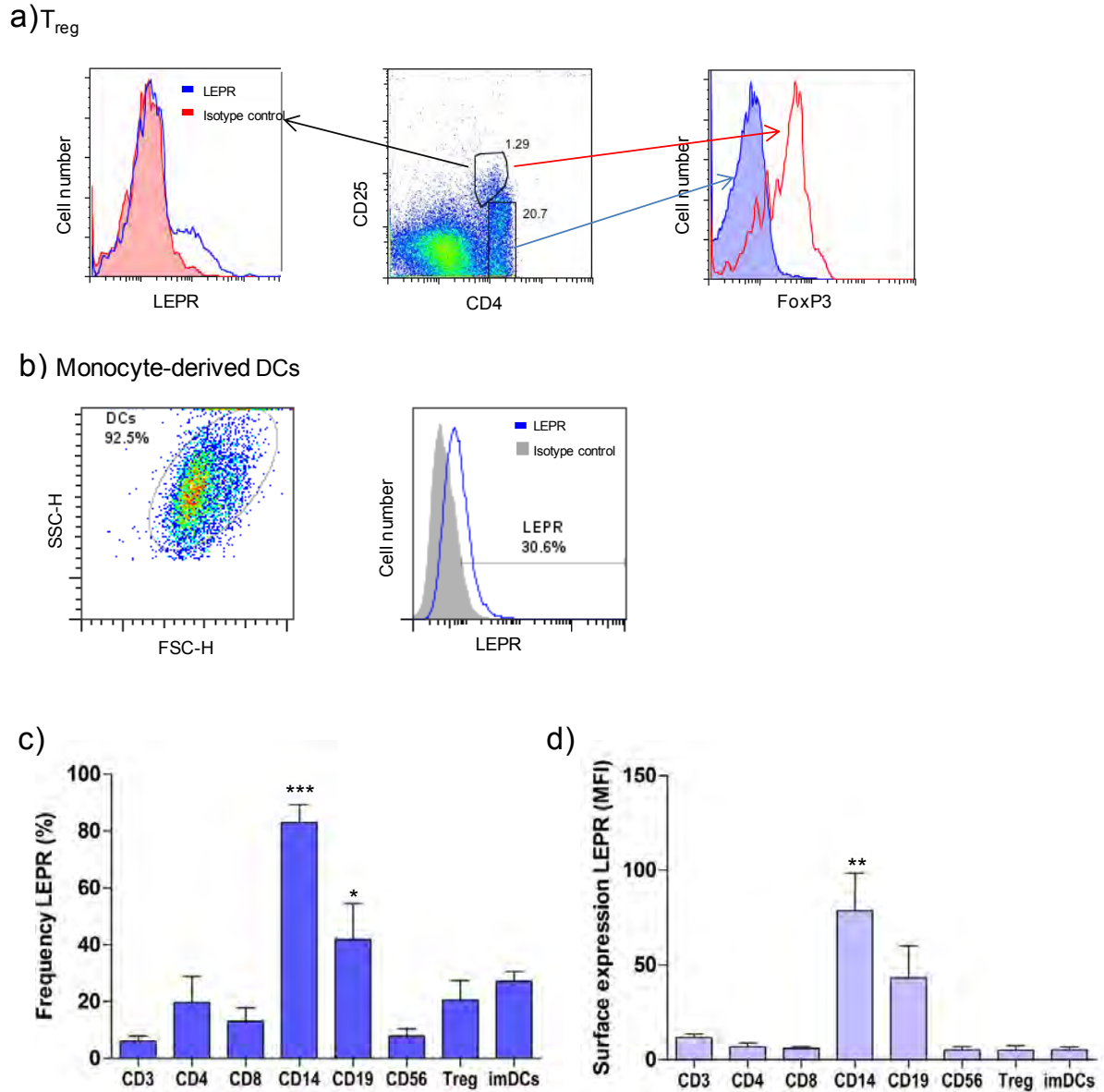


Figure 3-4: Distribution of LEPR expression on PBMC subpopulations

(a) Treg were identified based on their CD4⁺ CD25⁺ and FoxP3⁺ expression. (b) Monocytes-derived DCs were generated and LEPR expression was measured by flow cytometry. (c) The percentage of LEPR⁺ (FL1⁺) cells and (d) the MFI were determined on PI, FSC-H/SSC-H and subsets gated cells. Data are represented as mean±SEM and were analysed using one-way ANOVA and Bonferroni's multiple comparisons post-test. Pool of at least three experiments. *p≤0.05; **p≤0.01; ***p≤0.001.

2.1.3. *LEPR expression on T lymphocytes upon activation*

We went on to determine the effect of T cell activation on the expression of LEPR, using PHA as a T cell stimulator. LEPR expression was found to be slightly but not significantly increased after PHA stimulation for five days on all T lymphocytes, and both CD4⁺ and CD8⁺ T lymphocytes populations (**Figure 3-5**). The degree of T cells activation was confirmed with the increase in size and granularity observed on the forward/side scatter dot plots and the increase of CD25 expression, commonly up-regulated upon T cell stimulation (**Figure 3-5a**). It appears that the LEPR up-regulation following stimulation with PHA is higher for CD4⁺ T cells than for CD8⁺ T lymphocytes.

2.2. *Expression of leptin by PBMC*

DeRosa *et al.* have previously reported leptin expression by Treg using confocal microscopy and western blot experiments. Our aim here was to examine the expression of leptin in PBMC.

2.2.1. *At an RNA level*

We first analysed leptin expression by PBMC using conventional PCR. This method allowed detection of amplicons for leptin at the expected size of 180pb in both pre-adipocytes (positive control) and PBMC (**Figure 3-6a**). The absence of a band in the RT-ve and water negative controls shows the specificity of the reaction.

2.2.2. *At a protein level*

We went on to attempt detection of leptin at a protein level, using immunoblotting and flow cytometry. Immunoblotting of PBMC lysate revealed a light band at 16kDa, consistent with the predicted molecular weight of leptin and the recombinant leptin size (**Figure 3-6b**). However, leptin could not be detected in serum samples. Our data suggests that leptin is produced in low quantity by PBMC. We then proceeded to examine expression by flow

cytometry. PBMC were fixed and permeabilised with the buffers used for FoxP3 staining. The anti-leptin antibody was used at 10µg/ml and detected with the donkey anti-mouse Alexa488 secondary antibody at 5µg/ml. Intracellular leptin was detected in whole PBMC (**Figure 3-6c**). The labelling specificity was confirmed by the absence of staining with the isotype control. These results indicate that PBMC are able to produce leptin themselves.

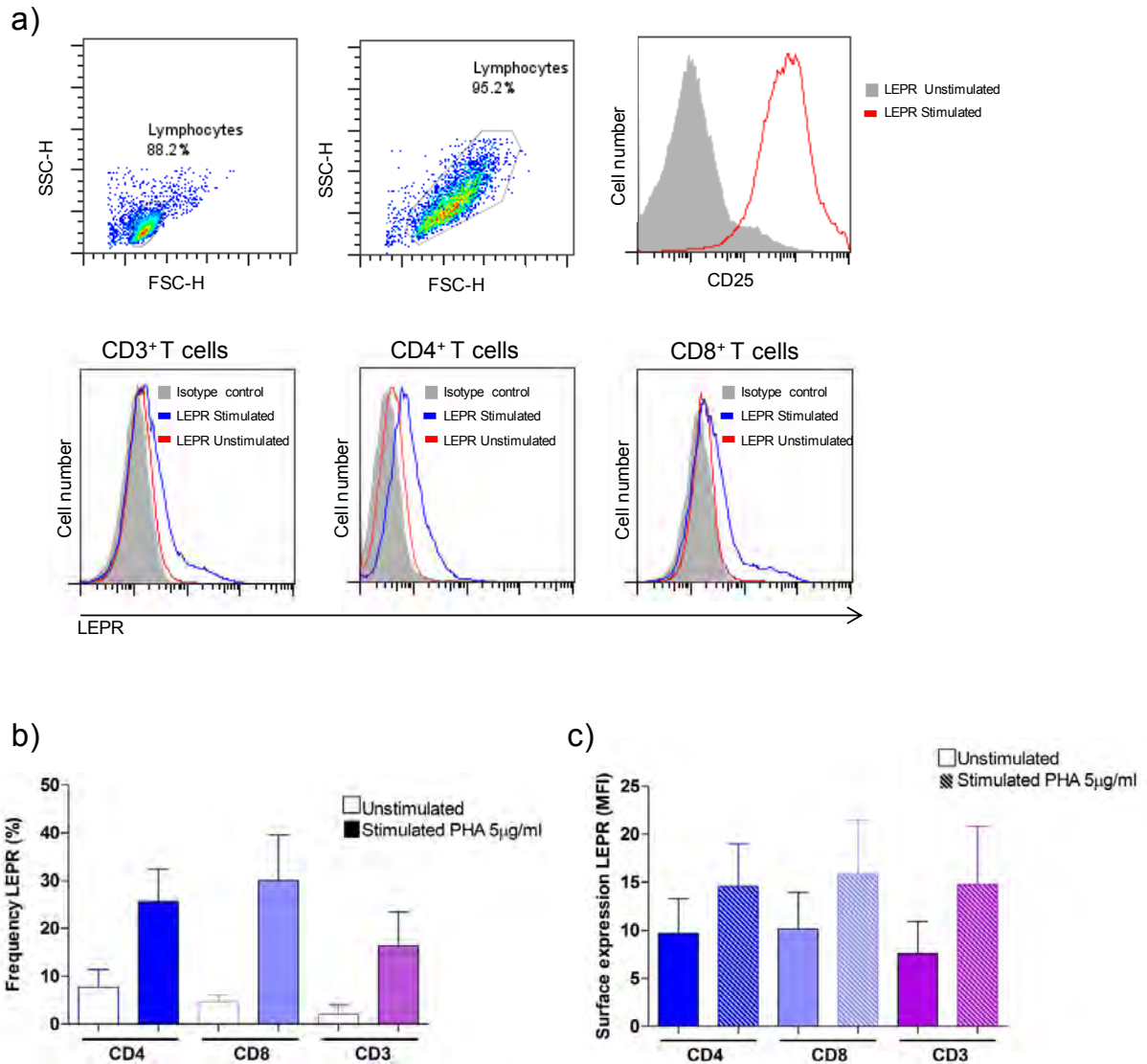


Figure 3-5: T cell activation up-regulates LEPR

PBMC were treated with 5µg/ml of PHA for 5 days. (a) Up-regulation of CD25 expression, as well as the increase in forward/side scatter on CD3 gated T cells demonstrates they have been stimulated. LEPR expression was measured on unstimulated and stimulated CD3 T cells and the CD4 and CD8 subsets and quantified as (b) a percentage of cells expressing the receptor and (c) with MFI. Data are shown as mean±SEM and were analysed using one-way ANOVA and Bonferroni's multiple comparisons post-test. Pool of at least three experiments. No significant differences were found.

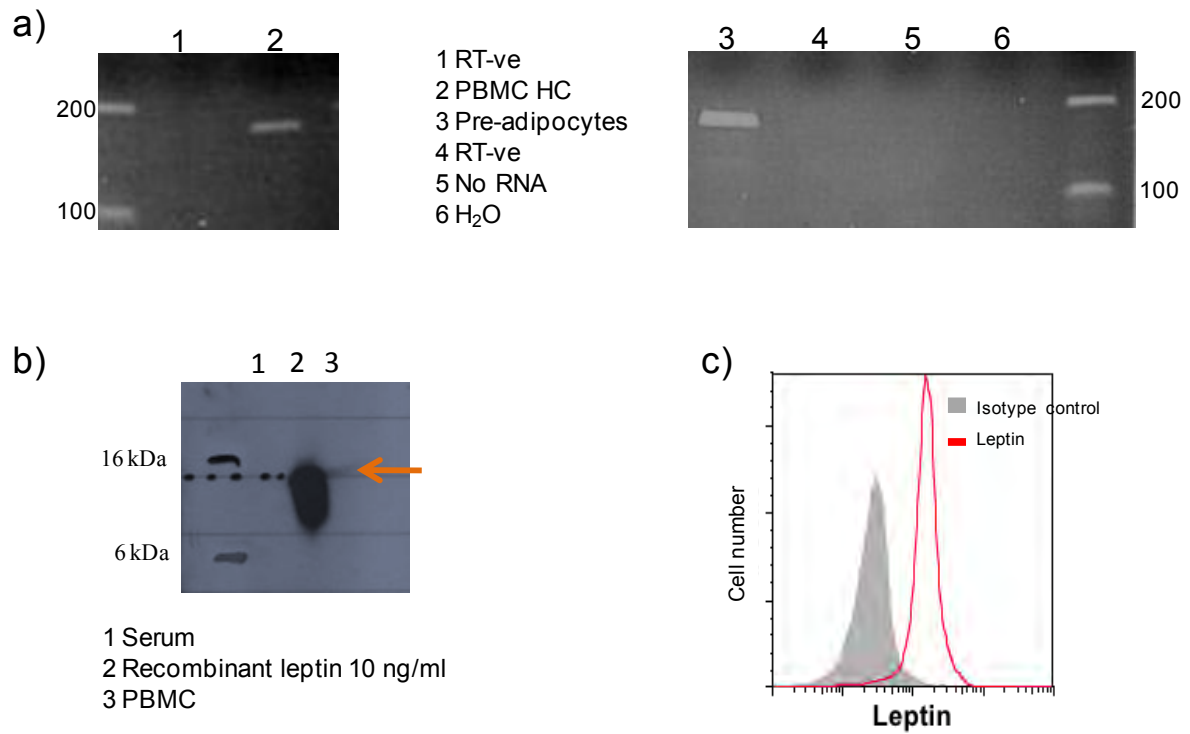


Figure 3-6: PBMC express leptin

(a) Leptin expression can be detected in total PBMC mRNA from healthy subjects by RT-PCR. RT-ve and water controls show no evidence of gDNA contamination in the samples. Pre-adipocytes mRNA was used as positive controls. The predicted amplicon size for leptin was 180bp. (b) Leptin was detected in PBMC lysate by western blot and (c) on fixed/permeabilised PBMC by flow cytometry. Data are representative of two experiments.

2.3. Effect of T1D on leptin and leptin receptors expression on PBMC

Leptin is known to modulate the function of leukocytes in both innate and adaptive systems in addition to its other pleiotropic effects. For these reasons, we aimed to identify any potential difference of LEPR and leptin levels in T1D compared to healthy controls (HC).

2.3.1. LEPR expression on PBMC, lymphocytes and monocytes from T1D, HC and T2D subjects at a protein level.

We recruited 49 Caucasian male diabetic subjects (T1D and T2D) from the Diabetes Clinics of the Birmingham University Hospital as well as 19 Caucasian male subjects matched for age with T1D patients. Clinical parameters of HC, T1D and T2D subjects are described in **Table 3-1**. We found no significant differences for age, body mass index (BMI), and waist/hip ratio (WHR) between HC and T1D subjects. However, these parameters were significantly higher for T2D subjects compared to HC and T1D.

Peripheral blood was collected from each subjects, serum was isolated and frozen at -80°C. The day after collection, PBMC were isolated, labelled and analysed by flow cytometry. LEPR expression was estimated by MFI on PI and forward/side scatter gated cell population. MFI was calculated on LEPR positive population with limits determined on the isotype control.

This T1D study revealed that T1D subjects exhibit significantly higher expression of LEPR on whole PBMC, lymphocytes and monocytes compared to HC and T2D subjects (**Figure 3-7, 8**). However, there were no significant correlations between LEPR expression and age, BMI, HbA1c and eGDR levels in T1D.

	HC	T1D	T2D
n	19	24	21
Age (years)	29.17 ± 6.40	34.84 ± 10.17	54.80 ± 11.45*
BMI (Kg/m²)	25.39 ± 2.45	24.43 ± 3.43	35.33 ± 7.31*
WHR	0.89 ± 0.06	0.90 ± 0.09	1 ± 0.08*
Duration of diabetes (years)	NA	13.44 ± 9.76	9.65 ± 6.81
HbA1c (%)	NA	9.08 ± 1.52	8.85 ± 1.93
Insulin dose (total daily/Kg)	NA	0.86 ± 0.26	NA
eGDR (mg/Kg/min)	NA	6.60 ± 6.78	NA
Systolic BP (mmHg)	NA	137.13 ± 18.83	140.96 ± 14.81
Diastolic BP (mmHg)	NA	84.25 ± 9.71	85.12 ± 8.99
Total Cholesterol (mmol)	NA	4.38 ± 0.56	4.09 ± 0.94
Triglycerides (mmol)	NA	1.58 ± 1.04	2.34 ± 1.41

Table 3-1: Clinical parameters of study patients

Data are represented as Mean ± SD and analysed using t-test. No significant differences for age, BMI and WHR were found between HC and T1D subjects. Age, BMI and WHR were significantly higher for T2D subjects than HC and T1D (*). No significant differences were found for duration of diabetes, glycated haemoglobin (HbA1c), systolic and diastolic blood pressure (BP), cholesterol and triglycerides between T1D and T2D.

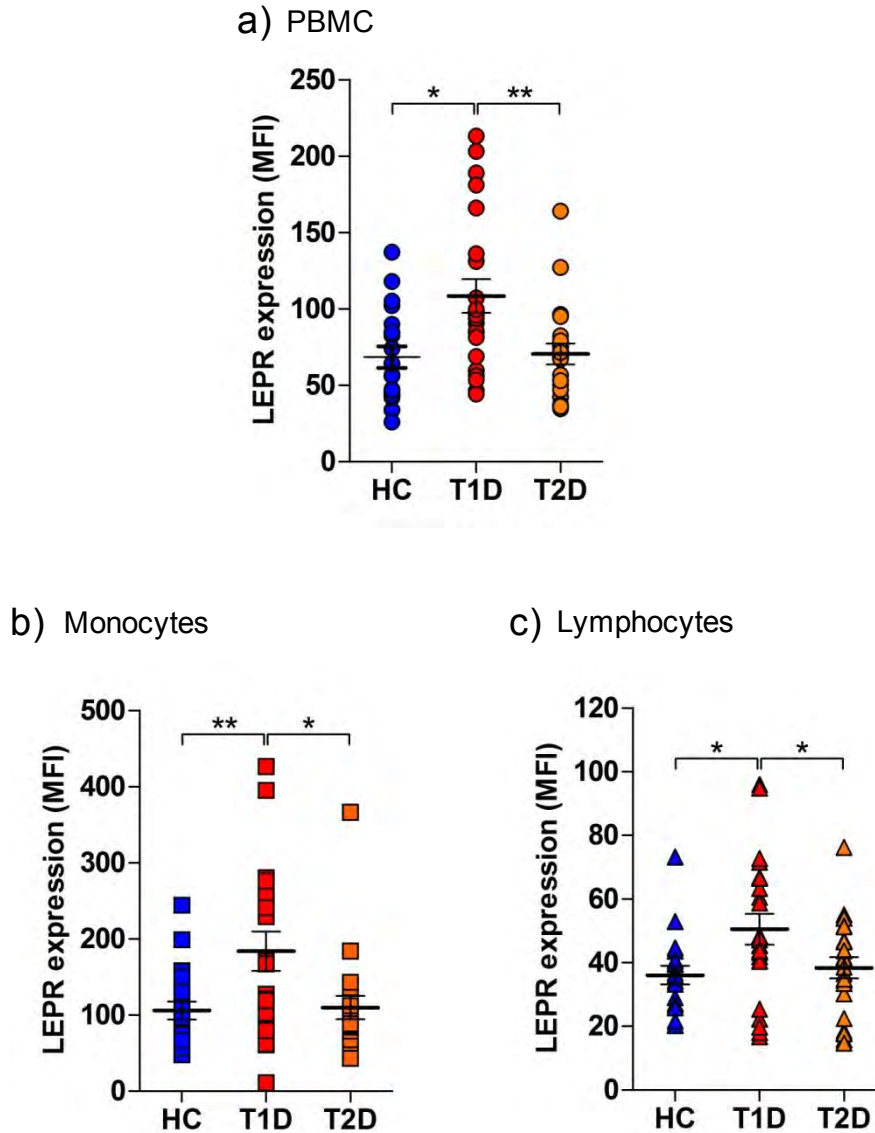


Figure 3-7: LEPR expression on PBMC from HC (blue), T1D (red) and T2D (orange) subjects by flow cytometry

(a) LEPR expression is higher in T1D subjects in whole PBMC, (b) lymphocytes and (c) monocytes subpopulations compared to HC and T2D subjects. Data are shown as Mean Fluorescence Intensity (MFI) \pm SEM and analysed using non-parametric Mann-Whitney t-test as some data test failed the Kolmogorov normality tests (meaning it does not follow Gaussian distribution). * $p \leq 0.05$, ** $p \leq 0.01$.

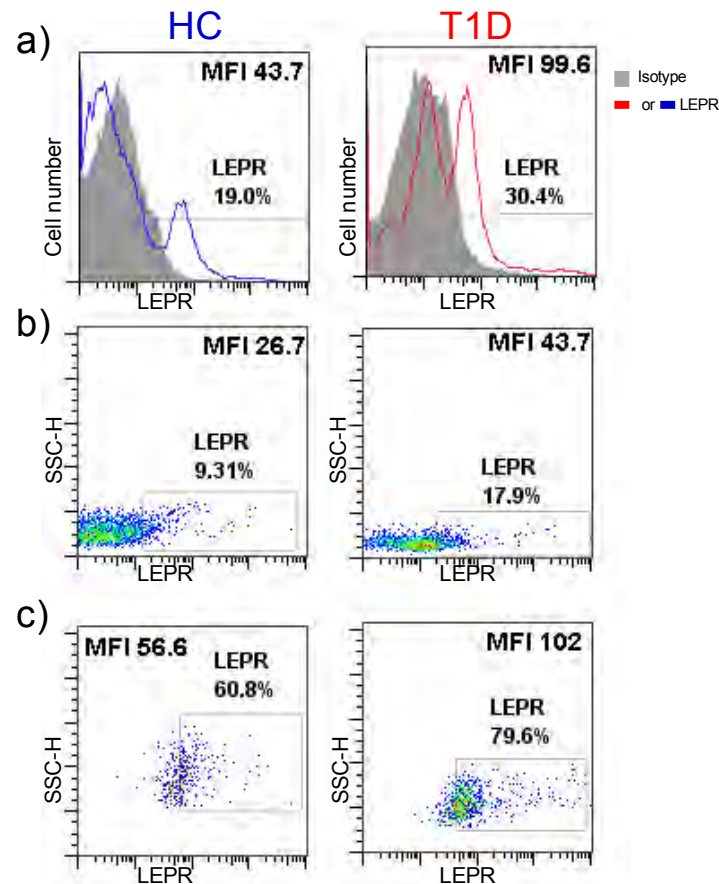


Figure 3-8: Representative histograms and dot plots of LEPR expression in HC and T1D (a) PBMC, (b) lymphocytes and (c) monocytes were gated as previously described. Gates were set on the isotype control (solid grey) and LEPR expression was determined by calculation of the MFI on the LEPR positive population. Gates show the percentage of LEPR positive cells, which is also higher in T1D on PBMC, lymphocytes and monocytes.

2.3.2. *sLEPR and lLEPR gene expression on PBMC from T1D, HC and T2D subjects*

We then analysed the expression of both *lLEPR* and *sLEPR* in HC, T1D and T2D using q-PCR. We confirmed that PBMC from T1D display higher relative gene expression of *LEPR* compared to HC and T2D subjects. Using sequence specific primers for the long and short isoforms of *LEPR*, we showed that the difference in *LEPR* expression was due to a significantly higher expression of the long but not the short isoform of *LEPR* (**Figure 3-9a, b**). We characterised that *lLEPR* tend to be predominant on PBMC from T1D subjects. However, this is different in HC and T2D subjects, as *sLEPR* isoforms is predominant in these patients (**Figure 3-9c, d, e**). Moreover, we revealed a positive correlation between *LEPR* expression at a protein level with *lLEPR* at mRNA level, but not for *sLEPR*, using linear regression analysis (**Figure 3-10**).

2.3.3. *Circulating leptin levels in health and diabetes*

Finally, we assessed concentrations of leptin in serum using immunoblotting. Leptin could not be detected using this method even in undiluted serum samples (**Figure 3-11a**). Only unspecific bands at 30 and 60kDa were observed in the serum lanes. The presence of a band at 16kDa in the recombinant leptin lane (lane 2) and its blocking (lane 1) demonstrate that the appropriate controls have worked.

Because immunoblotting was not sufficiently sensitive to detect leptin in serum, we assessed circulating leptin concentrations by ELISA. This assay revealed no significant difference in leptin in T1D compared to HC subjects (**Figure 3-11b**). However, significant higher circulating level of leptin was noticed in T2D subjects. In all subjects, leptin significantly and positively correlated with BMI and insulin dosage in T1D (**Figure 3-12**).

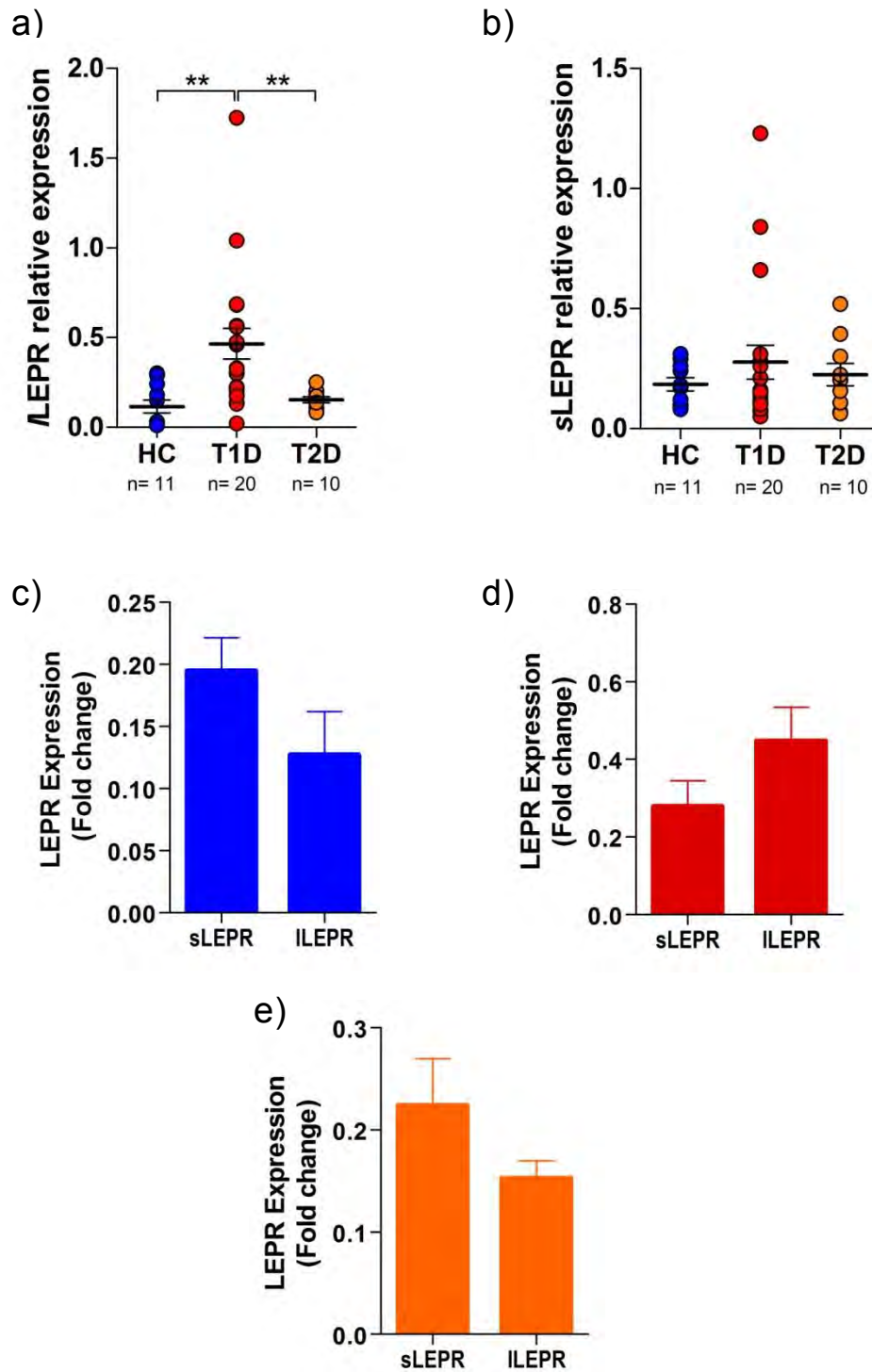


Figure 3-9: $sLEPR$ and $lLEPR$ expression on PBMC

(a) Expression of the $lLEPR$ and (b) $sLEPR$ was quantified and compared in (c) HC, (d) T1D and (e) T2D subjects. Relative $LEPR$ expression was quantified by real-time quantitative PCR using $HPRT1$ as endogenous control. Data were analysed using Mann-Whitney t-test. ** $p \leq 0.01$.

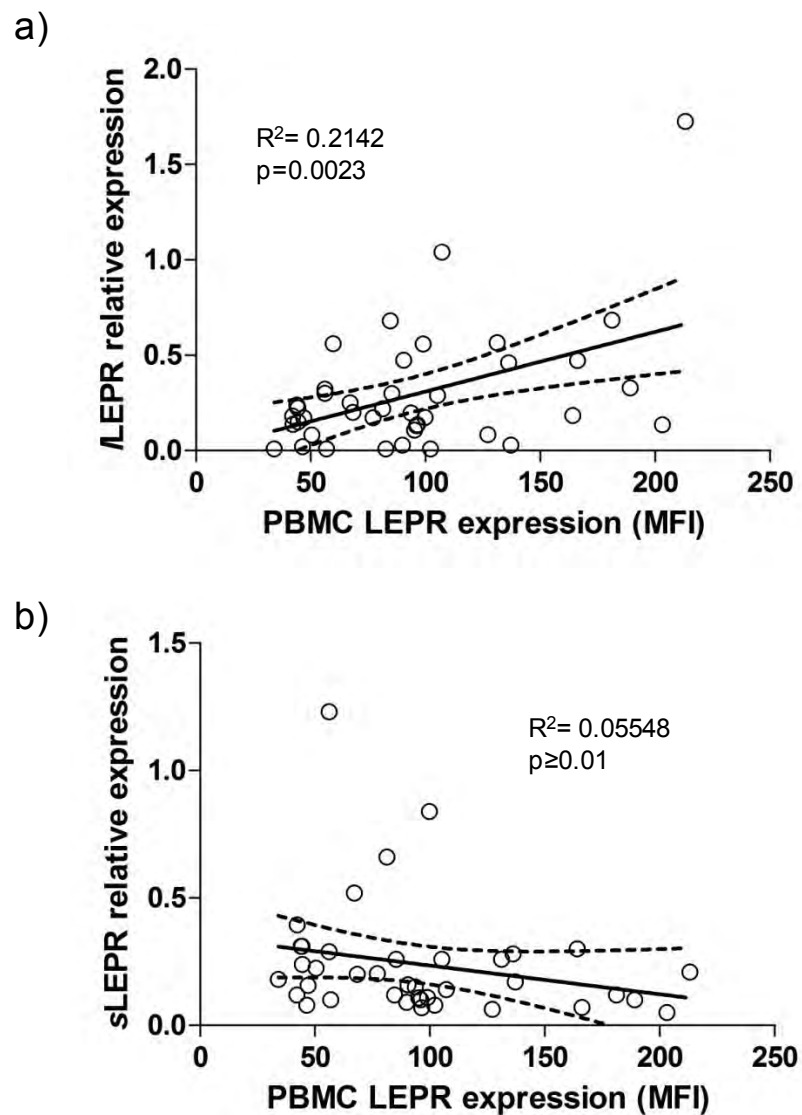


Figure 3-10: Correlation between LEPR expression at a protein level and mRNA level
Correlations between mRNA level of (a) Δ LEPR and (b) sLEPR with protein expression of LEPR were determined using linear regression analysis, showing correlation for the Δ LEPR only.

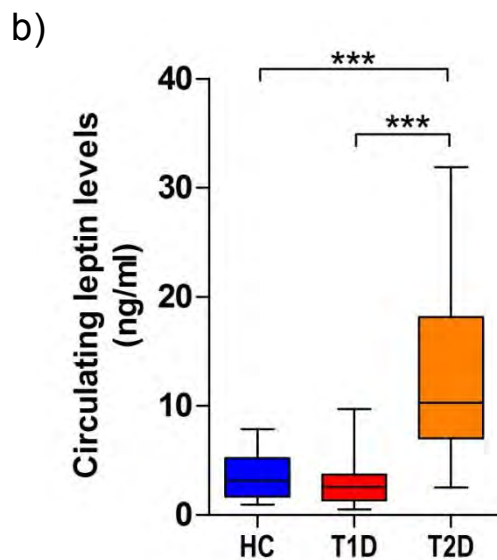
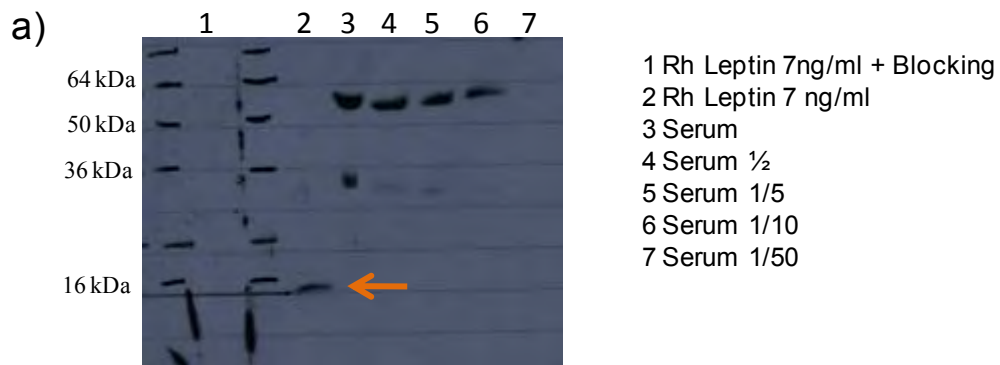


Figure 3-11: Circulating leptin levels in health and disease

Circulating leptin in HC, T1D and T2D subjects' were measured by (a) Western Blot and (b) ELISA. Data were analysed using Mann-Whitney t-test. *** $p \leq 0.001$.

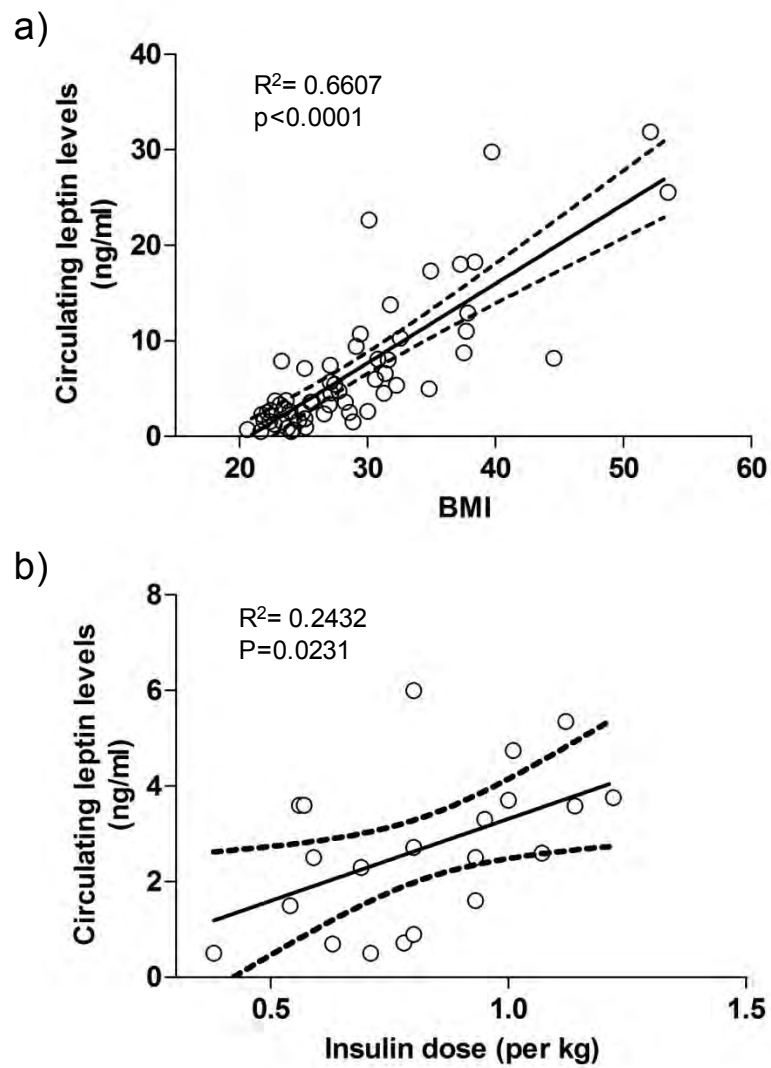


Figure 3-12: Correlations between circulating leptin and BMI or insulin dose

(a) Correlation between leptin levels and BMI was determined by linear regression analysis in all subjects from the HC, T1D and T2D groups (b). Correlation between leptin levels and insulin dose was determined by linear regression analysis in the T1D group.

3. Discussion

We demonstrate that LEPR is highly expressed on monocytes, B cells and Treg and to a lower extent on T cells. We show for the first time that subjects with T1D express higher levels of LEPR on whole PBMC, monocytes and lymphocytes. The functional role of this remains unclear and will be examined in the next chapter.

- **Characterisation of leptin and LEPR on PBMC in health**

LEPR expression on whole PBMC was confirmed by both conventional PCR and flow cytometry in healthy controls. PCR allowed us to measure expression of both the short and long form leptin receptors isoforms using intron-spanning primers designed in the intracellular region of LEPR. The epitope recognised by the anti-LEPR antibody is located on the extracellular N-terminal region, and the difference between the two isoforms is located in the C-terminal intracellular region. Thus, the antibody does not allow us to distinguish between *s*LEPR and *l*LEPR by flow cytometry (Bennett *et al.*, 1996). Conventional PCR allowed us to confirm the expression of *s*LEPR and *l*LEPR on whole PBMC, T cells and monocyte-derived DCs. The expression was not due to gDNA contamination as we used intron-spanning primers and the controls without reverse-transcriptase are clear. Flow cytometry analysis allowed us to characterise the expression of LEPR on PBMC sub-populations. Previous studies identified LEPR on whole PBMC, monocytes, DCs, macrophages, NK cells and CD4⁺ or CD8⁺ T cells (Martin Romero *et al.*, 2000; Lord *et al.*, 1998 and 2002; Zarkesh-Esfahani *et al.*, 2001; Mattioli *et al.*, 2005). However, the expression of LEPR on these cells was not quantified and compared, and most studies have looked in mice (Zhao *et al.*, 2003; Siegmund *et al.*, 2004; Papathanassoglou *et al.*, 2006; De Rosa *et al.*, 2007). In agreement with the literature, we found LEPR on these subsets using a monoclonal antibody optimised for flow cytometry. LEPR is highly expressed on CD14⁺ monocytes, and

slightly less on CD19⁺ B cells and monocytes-derived DCs followed by expression on Treg. LEPR expression was found on CD4⁺ and CD8⁺ T cells at a lower extent. We found no expression of LEPR on NK cells in contradiction with other studies in mice and humans (Zhao *et al.*, 2003).

Quantification of LEPR on each subset was measured as the frequency of cells expressing the receptors as well as surface expression by MFI. Both ways show the same profile of expression. Additionally, specificity of LEPR labelling was confirmed using an isotype control and by blocking the antibody with recombinant LEPR prior staining. This confirms the reliability of our technique to quantify LEPR expression by flow cytometry unlike in other studies that have not used isotype or blockade (Papathanassoglou *et al.*, 2006; De Rosa *et al.*, 2007; Martin Romero *et al.*, 2000; Mattioli *et al.*, 2005; Zarkesh-Esfahani *et al.*, 2001).

We show that LEPR expression is increased on stimulated CD4⁺ and CD8⁺ T cells with regard to frequency of LEPR positive cells, and also surface expression characterised by MFI. This was also reported by others with mice and human lymphocytes (Papathanassoglou *et al.*, 2006; Sanchez-Margalet *et al.*, 2002). In these studies, LEPR expression is also increased on CD4⁺ and CD8⁺ T cells after anti-CD3 or PHA stimulation as well as on mouse B cells and macrophages. These data suggests that LEPR is a marker of cell activation and is in agreement that T cells are sensitive to leptin only in stimulation conditions (Lord *et al.*, 1998).

Interestingly, DeRosa *et al.* revealed that CD4⁺ T lymphocytes and T_{reg} produce leptin. In this study, the authors detected leptin at a protein level by immunoblotting and confocal microscopy (De Rosa *et al.*, 2007), however, no other studies have confirmed this to date. We therefore aimed to quantify leptin expression in PBMC at both RNA and protein level. We confirmed low leptin expression in PBMC by conventional as well as immunoblotting and

flow cytometry. However, problems of non-specific intracellular binding can occur, particularly when using a polyclonal antibody in flow cytometry and western blotting. Thus, the specificity of the labelling would optimally require to be confirmed by blocking the leptin antibody with recombinant leptin beforehand. Although flow cytometry and classic western blotting are commonly used to identify and quantify protein expression, in this case these are not optimal to prove production of leptin by PBMC. Leptin production by PBMC should be determined using immunoprecipitation followed by mass spectrometry analysis. This method would confirm leptin presence in PBMC in a qualitative manner.

- **Effect of T1D on leptin and LEPR expression**

A number of studies have shown that leptin deficiency is associated with protection against inflammatory and autoimmune disease. Leptin-deficient mice do not develop nephrotoxic nephritis and experimental arthritis as well as atherosclerosis and similarly in mice lacking LEPR (Tarzi *et al.*, 2004; Busso *et al.*, 2002; Taleb *et al.*, 2007), T cell-mediated hepatitis (Faggioni *et al.*, 2000), chronic intestinal inflammation (Mykoniatis *et al.*, 2003) and EAE (Sanna *et al.*, 2003; Matarese *et al.*, 2001). Similarly, leptin neutralisation in mice protects from development of EAE (DeRosa *et al.*, 2006). However, leptin deficiency is associated with reduction in thymus size and frequency of immune cells causing defects in T cell mediated immunity (Chandra *et al.*, 1980; Fernandes *et al.*, 1978). Therefore, absence of leptin predisposes to infections and this was not investigated in these studies.

Because leptin has been shown to accelerate the progression of T1D in the NOD mouse model and other autoimmune disease models, we believe that leptin has a potential involvement in the development of T1D in humans (Matarese *et al.*, 2002). We thus aimed to identify any difference in circulating leptin levels and LEPR expression on PBMC in T1D. Contrary to what we expected and to the current literature, T1D is not associated with high

circulating leptin serum levels. All our patients with T1D were matched with our healthy controls for ethnicity, gender, BMI, age and WHR in contrast to the studies that observed a difference. Within the T1D group, all patients were matched for ethnicity, gender, BMI, age, WHR, HbA1c, duration of diabetes, insulin treatment, lipid profile and prevalence of vascular complications. This indicates the reliability of the differences found between the groups, in opposition to previous studies lacking this level of control (Kiess *et al.*, 1998; Lunaet *al.*, 1999; Hanaki *et al.*, 1999; Fluck *et al.*, 1999; Kirel *et al.*, 2000; McCormick *et al.*, 2001; Soliman *et al.*, 2002; Bideci *et al.*, 2002; Sandoval *et al.*, 2003; Lo *et al.*, 2004; Gilliam *et al.*, 2006). In addition, studies have shown that leptin levels vary according to a diurnal cycle (Sandoval *et al.*, 2003). This means that studies investigating circulating concentrations of leptin should be consistent for the time of sampling. This has not been mentioned in the studies considered. In our study, blood sampling was always consistently drawn in the afternoon and serum was isolated straight away and stored at -80°C for all patients and HC.

We believe that in matched subjects, leptin levels are unchanged in T1D compared to HC. Nevertheless, much of the destructive autoimmune process occurs before the onset of clinical diabetes. Leptin levels may possibly be high before the onset of T1D. Supporting this hypothesis, a surge in serum leptin occurs at preclinical stage, before the onset of hyperglycaemia in the NOD mouse model (Matarese *et al.*, 2002) as well as before development of EAE (Sanna *et al.*, 2003). To our knowledge, no studies in humans have investigated leptin levels in pre-T1D. Leptin levels have only been measured in long lasting T1D as well as some newly diagnosed patients. However, the data are highly contradictory and it is therefore difficult to draw a conclusion. Most of the studies show higher levels in long duration of disease in correlation with insulin dosage. Even though, we found unchanged leptin levels in our matched cohort, we agree that leptin levels positively correlate with insulinisation. Studies in pre-T1D have shown that insulin resistance is a risk factor for

developing T1D (Furlanos *et al.*, 2004). If leptin levels are shown to be higher in pre-T1D, association between IR and development of T1D could well be contributed to by leptin. Additionally, study in pre-T1D would show leptin levels independently of insulinisation, as these patients are generally not on insulin.

We also show that circulating leptin concentrations in T2D are higher compared with HC and T1D. Leptin levels positively correlated with BMI and WHR in all groups. The BMI of our group of patients with T2D being significantly higher than HC and T1D, this explains the rise in leptin levels in this group. This is in agreement with the current literature showing higher leptin levels in obese T1D and also T2D (Verroti *et al.*, 1998).

In T1D, we found that LEPR is highly expressed on circulating immune cells compared to HC and T2D. On the other hand, in T2D, LEPR expression is not higher showing that the rise observed in T1D is specific to the disease and not to high blood level of glucose. Alternatively, reduced expression of LEPR on PBMC from T2D compared to T1D could be due to the high leptin circulating levels. Indeed, leptin has been shown to downregulate the expression of the *l*LEPR on hepatocytes (Liu *et al.*, 2005), neuroblastoma cells (Hikita *et al.*, 2000) and in the brain (Mitchell *et al.*, 2009) but there are no evidence on PBMC.

We found no significant correlations between LEPR on PBMC, monocytes and lymphocytes and all the parameters measured in the study except for a negative association between LEPR and BMI. This is in agreement with previous findings showing a significantly lower expression of both *s*LEPR and *l*LEPR in overweight versus lean subjects (Tsiotra *et al.*, 2000). This observation could explain why expression of LEPR in T2D is lower than in the T1D group. Indeed, T2D might be associated with higher LEPR expression but because these patients tend to have high BMI, this compensates the receptor levels on PBMC. Therefore, it would be worth examining the expression of LEPR in leaner patients with T2D.

Additionally, we observed a significant correlation between the levels of LEPR on PBMC at a protein level and *l*LEPR gene expression. Interestingly, this correlation was not found for the short isoform. This implies that only the functional long form of the receptor is modulated in T1D and therefore suggests a potential functional significance as *l*LEPR only seems to induce signalling cascades mediating leptin actions (Chen *et al.*, 1996; Lee *et al.*, 1996; Cioffi *et al.*, 1996).

We did not find any correlation between expression of LEPR on PBMC and subsets and index of insulin resistance in our T1D cohort, which would support our general working hypothesis. Several explanations could account for this observation.

First, insulin resistance was measured by eGDR calculation in our cohort of T1D. Although this method has been validated for adult patients with T1D (Williams *et al.*, 2000), the most common and accurate technique is the euglycaemic hyperinsulinaemic clamp because it measures direct response of insulin to glucose *in vivo*. Supporting this, we found that the levels of LEPR on PBMC in T2D are not different from HC. Indeed, T2D is a condition strongly associated with insulin resistance. Measurement of insulin resistance was not accomplished in these patients and the eGDR method is not validated in this disease. On the other hand, the difference could only be specific to the autoimmune context of T1D. Indeed, independently of insulin resistance, leptin has widely been reported as a modulator of immune function. This is again supported by the level of LEPR in T2D that has no association with autoimmunity. Furthermore, surveys have reported a negative correlation between insulin resistance and LEPR expression in adipose tissue (Huan *et al.*, 2003). Similarly, over-expression of LEPR in the hypothalamus improves peripheral insulin sensitivity in skeletal muscle (German *et al.*, 2009). Finally, high levels of LEPR could also be due to insulin treatment. For instance, insulin has been reported to induce up-regulation of LEPR in

neuroblastoma cells (Hikita *et al.*, 2000). However, we found no correlation between insulin dosage and LEPR levels in our cohort of patients.

We believe that high expression of LEPR on PBMC potentially makes them more responsive to the pro-inflammatory effects of leptin. This is supported by a study revealing that autoimmunity in the NOD mouse model is the consequence of T conventional hyper-responsivity rather than a Treg dysfunction (D'Alise *et al.*, 2008). We believe that this hyper-responsiveness could be partly attributed to the high leptin receptor expression and the subsequent leptin effect. Because T cells are hyper-responsive and Treg proliferation is limited, Treg themselves are not sufficient to efficiently regulate T cell responses. This potential mechanism, even if consistent with our findings in addition to previous studies, requires to be investigated in a T1D context.

Although our data in T1D is novel, studies have shown modulation of LEPR expression in other diseases. LEPR expression is decreased on PBMC from chronically-infected hepatitis B (HBV) and hepatitis C (HCV) patients, whilst leptin levels are decreased (Stefanou *et al.*, 2006). These data suggests that lower circulating leptin and LEPR expression contribute to the virus-mediated down-regulation of immune responses. Indeed, less receptor on PBMC reduces the ability of leptin to stimulate immune responses against the pathogen (Stefanou *et al.*, 2006). These studies show for the first time how crucial LEPR modulation of immune cells and how it correlates with the pro- or anti-inflammatory context. In addition, Frisullo *et al.* found higher expression of LEPR on PBMC from patients with relapsing multiple sclerosis (Frisullo *et al.*, 2007). The levels of LEPR on PBMC were restored in remitting patients. In addition, leptin levels were unchanged in relapsing patients compared to HC. This suggests that even though leptin concentrations are unchanged, PBMC are more sensitive to its action as they express more of the receptor (Frisullo *et al.*, 2004). These data and our study, in contrast with the infection model, suggests that higher levels of LEPR are

associated with up-regulation of pro-inflammatory immune responses and indicate that therapy targeting LEPR rather than leptin itself may be more appropriate for treatment of autoimmune diseases.

- **Conclusions**

Taken together these data demonstrate for the first time a difference in LEPR expression in T1D. LEPR is globally augmented at both protein and RNA level on PBMC, lymphocytes and monocytes from patients with T1D compared to matched controls and to patients with T2D. We propose the high expression on LEPR on immune cells contributes to the inflammation that characterises T1D.

4.CHAPTER 4- AN EXAMINATION OF THE EFFECTS OF LEPTIN ON IMMUNITY

1. Introduction

In this chapter, we aimed to demonstrate functional relevance for the higher expression of LEPR detected on PBMC from patients with T1D.

T cell proliferation and pro-inflammatory cytokine production have been reported to increase in the presence of leptin (Martin- Romero *et al.*, 2000; Lord *et al.*, 1998; Zarkesh-Esfahani *et al.*, 2001). However, these studies have used techniques such as thymidine incorporation or up-regulation of CD25 to quantify T cell proliferation. We wished to validate these results using alternative measures of proliferation.

Carboxyfluorescein (CFSE) is a highly permeable dye that labels intracellular molecules. CFSE allows measurement of T cell proliferation as it progressively halves between daughter cells following each division (Lyons *et al.*, 2000), a dilution that can be measured by flow cytometry. To our knowledge, no studies have looked at antigen-specific responses in presence of leptin. Furthermore, some of the published experiments were conducted in media containing bovine serum that contains leptin, which cross-reacts with human leptin receptors. We therefore aimed to validate these observations in serum-free conditions.

We also wished to examine the effect of leptin on antigen presenting cells (APCs) such as dendritic cells (DCs). Studies in humans have shown that leptin promotes production of pro-inflammatory cytokines by DCs with a concomitant decrease of IL-10 secretion and increases their survival and migratory capacities (Mattioli *et al.*, 2005 and 2008). These DCs polarise T cells towards a Th1 phenotype and are very efficient at activating CD8⁺ cytotoxic T cells (Mattioli *et al.*, 2008). Additionally, DCs from ObR deficient mice expressed lower co-stimulatory molecules and had decreased capacity to stimulate allogenic T cells (Lam *et al.*, 2006).

Ultimately, we developed interest for lymphocyte migration and how adipokines might influence their recruitment to the endothelium. Leptin has previously been linked to the promotion of leukocyte migration (Cadelfie-Chezet *et al.*, 2003; Gruen *et al.*, 2007; Mattioli *et al.*, 2005). Indeed, leptin has been identified as a chemoattractant for THP-1 monocytes and DCs using Boyden chamber assays (Gruen *et al.*, 2007; Mattioli *et al.*, 2008). However, more detailed information of the functions of leptin in regulating leukocyte recruitment is lacking and there is no data on the regulation of lymphocyte trafficking. Thus for leptin, the data are limited to experiments using cell lines in assays lacking realistic barriers for trafficking (i.e. endothelial cells).

Here, we aimed to measure the effect of leptin on the migration of peripheral blood lymphocytes (PBL) across endothelial cells. We chose human umbilical vein endothelial cells (HUVEC) as a model of endothelium. HUVEC have been widely used to study leukocyte recruitment in both static and flow conditions (Luscinskas *et al.*, 1989; Furie *et al.*, 1991; Luscinskas *et al.*, 1995; Bahra *et al.*, 1998; Luu *et al.*, 2003; Butler *et al.*, 2005; Sheikh *et al.*, 2005). HUVEC express classic endothelial markers such as CD31, von Willebrand factor (vWF), ICAM-1, VCAM-1 and E-selectin. In this system, we cultured HUVEC on clear plastic and stimulated them with TNF- α and IFN- γ for 24 hours prior to measurement of recruitment as described in Material and Methods (**Chapter 2, section 4.7.2**). In these conditions, TNF- α /IFN- γ stimulation allows up-regulation of E- and P-selectin, ICAM-1 and VCAM-1 (Bevilacqua *et al.*, 1989; Bahra *et al.*, 1998; Lidington *et al.*, 1999). HUVEC stimulation also induces surface presentation of chemokines such as CXCL9, 10 and 11 important for lymphocyte recruitment as well as IL-8, CXCL1, CXCL5, CCL2 and PAF important to support monocyte and neutrophil recruitment (Huber *et al.*, 1991; Kuijpers *et al.*, 1992; Beck *et al.*, 1999; Ding *et al.*, 2000; Piali *et al.*, 1998). The model is essentially that already reported by McGettrick *et al.* in which the stimulation of the interferon inducible

CXC chemokines (CXCL9-11) is essential for efficient lymphocyte recruitment (McGettrick *et al.*, 2009).

PBL were added to stimulated HUVEC for 6 minutes and recruitment measured using phase contrast video-microscopy in both static and flow conditions. This technique has been widely used to measure recruitment of T cells, neutrophils and monocytes. It allows identification of phase bright firmly adhered PBL recruited on the endothelium and phase dark PBL located underneath the endothelium and which have therefore achieved trans-endothelial migration (transmigration). The effect of leptin was measured by treatment of HUVEC with this agent over the 24 hours of TNF- α and IFN- γ stimulation, or by pre-treatment of PBL for one hour prior to addition to the HUVEC. This allowed the direct effects on HUVEC or PBL to be distinguished.

In this chapter, we intended to analyse the effect of leptin on T cell proliferation using the CFSE dilution technique with mitogenic and antigen-specific stimulators. We then sought to measure production of pro and anti-inflammatory cytokines by T cells and aimed to analyse the effect of leptin on monocyte-derived imDCs phenotype and function. Finally, we investigated the effect of leptin on lymphocyte recruitment to the endothelium.

2. Results

2.1. *Leptin has no effect on T cell proliferation*

T cell proliferation assays were conducted in serum-free media, and cell proliferation measured by flow cytometry. Live cells were gated by PI exclusion labelled cells and CFSE dilution quantified by gating on CD3⁺ or CD4⁺CD25⁻ T cells (**Figure 4-1**). Using these conditions, the addition of leptin in physiological and supra-physiological doses did not influence mitogenic (PHA), CD3/CD28, anti-CD3, or antigen (Tetanus Toxoid protein- TT protein) induced proliferation of PBMC from healthy subjects (**Figure 4-2**, **Figure 4-3**, **Figure 4-4** and **Figure 4-5** respectively).

Previous studies have measured the effect of leptin on purified $CD4^+$ T cell proliferation. We therefore sorted healthy controls' $CD4^+CD25^-$ T cells and monocytes for co-stimulation, using anti-CD3 or Tetanus toxoid protein as stimulators. T cell and T cells+monocytes, or T cells+anti-CD3 or Tetanus toxoid protein were used as unstimulated controls. Once again, the addition of recombinant leptin did not influence T cell proliferation (**Figure 4-6 and Figure 4-7**).

In conclusion, the current literature showing that T cell proliferation is increased in the presence of leptin cannot be reproduced in our hands using serum-free media.

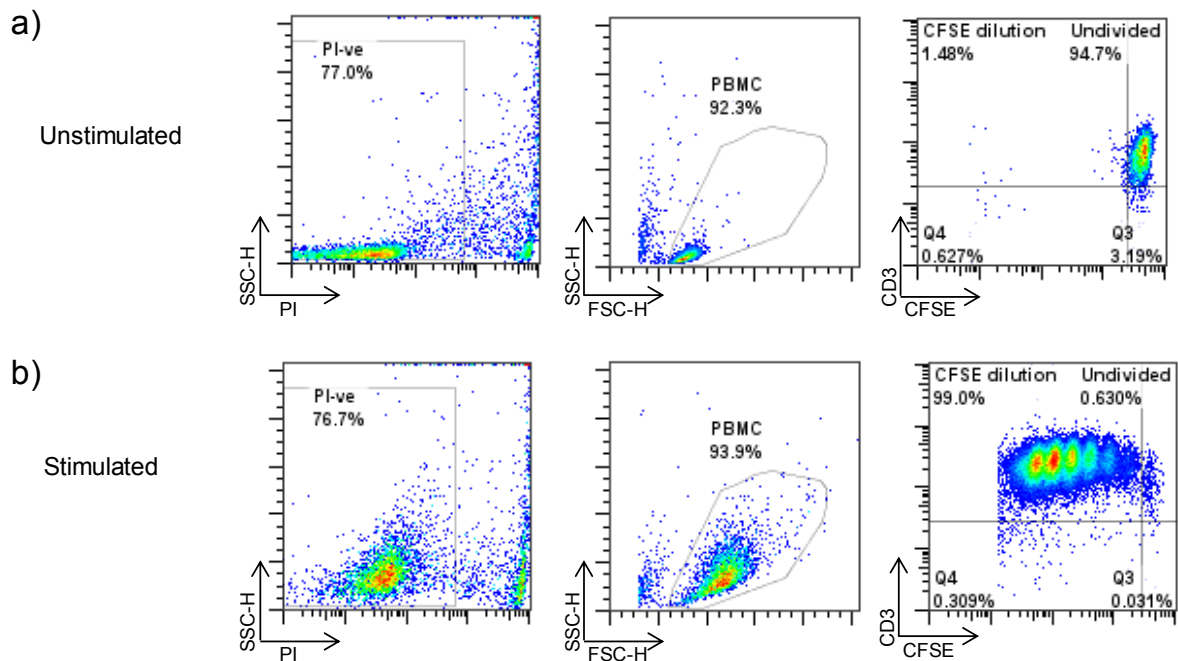


Figure 4-1: Gating strategy for measuring T cell proliferation using flow cytometry

(a) CFSE labelled PBMC were isolated from the peripheral blood and cultured for 5 days and (b) stimulated with PHA ($3\mu\text{g/ml}$). Live cells were gated on PI staining and lymphocytes on forward/side scatter. Stimulation induces a dramatic increase in granularity and size. Unstimulated cells did not proliferate as indicated by the absence of CFSE dilution. PHA stimulation resulted in a dilution of CFSE. Data are representative of at least three independent experiments.

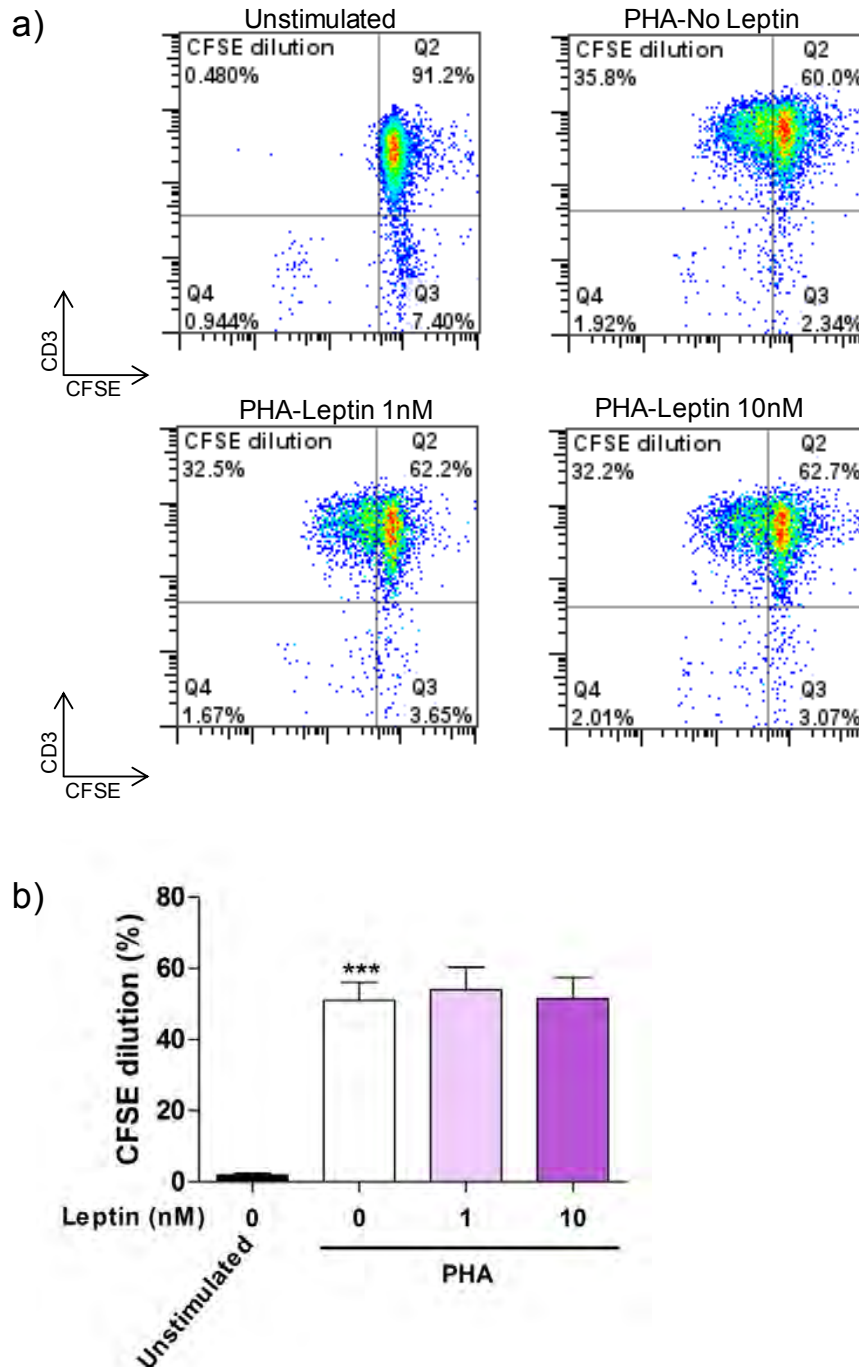


Figure 4-2: Leptin has no effect on PHA-induced T cell proliferation

(a) PBMC were isolated from the peripheral blood, labelled with CFSE and stimulated with $3\mu\text{g/ml}$ PHA in absence or presence of leptin at 1 and 10nM for three days. PHA induced T cell proliferation was measured by CFSE dilution. (b) T cell proliferation was significantly induced by PHA but no significant differences were found with leptin at 1 or 10nM. Data are mean \pm SEM and were analysed using one-way ANOVA and Bonferroni's multiple comparisons post-test. Pool of at least three experiments. *** $p\leq 0.001$.

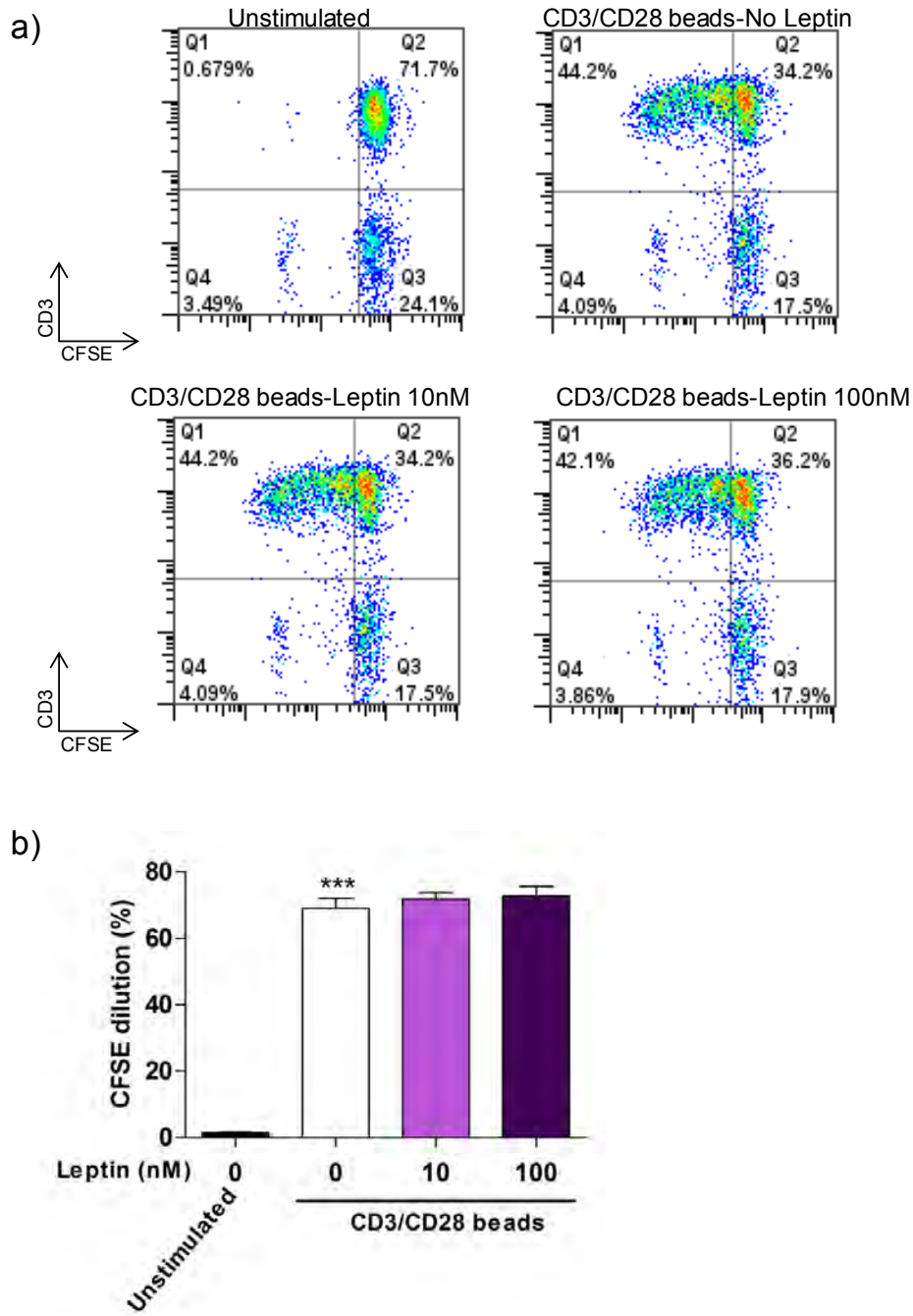


Figure 4-3: Leptin has no effect on CD3/CD28 beads-induced T cell proliferation

(a) PBMC were isolated from the peripheral blood, labelled with CFSE and stimulated with CD3/CD28beads (8:1, cells to bead) in absence or presence of leptin at 10 and 100nM for three days. CD3/CD28 beads induced T cell proliferation was measured by CFSE. (b) T cell proliferation was significantly induced by CD3/CD28 beads, but this was not affected by the addition of leptin. Data are mean \pm SEM and were analysed using one-way ANOVA and Bonferroni's multiple comparisons post-test. Pool of at least three experiments. *** $p \leq 0.001$.

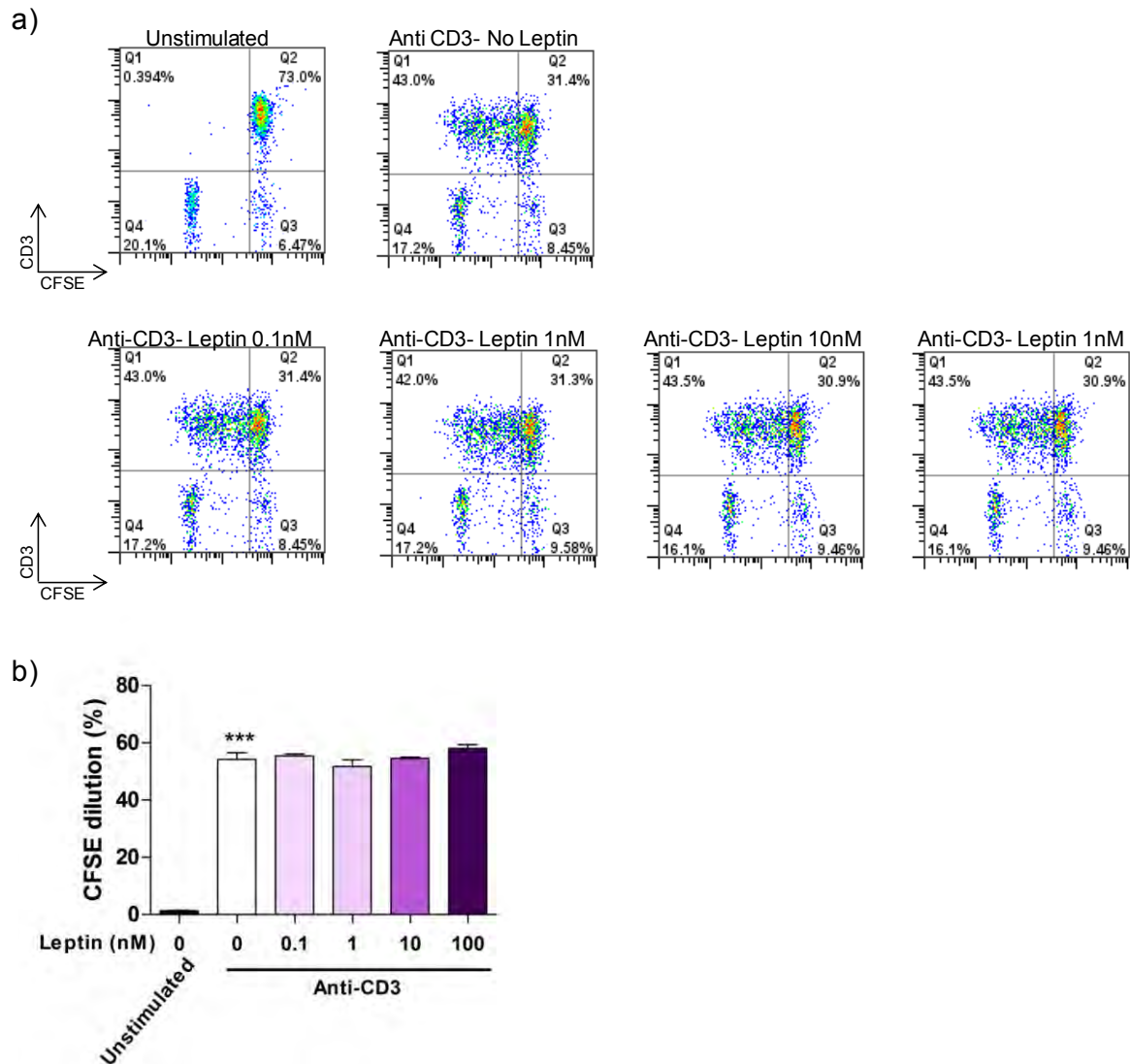


Figure 4-4: Leptin has no effect on Anti-CD3-induced T cell proliferation

(a) PBMC were isolated from the peripheral blood, labelled with CFSE and stimulated with 0.5 μ g/ml of anti-CD3 in absence or presence of leptin at 0.1 to 100nM for three days. Anti-CD3 induced T cell proliferation was measured by CFSE dilution. (b) T cell proliferation was significantly induced by anti-CD3 but no significant differences were found with leptin. Data are mean \pm SEM and were analysed using one-way ANOVA and Bonferroni's multiple comparisons post-test. Pool of at least three experiments. *** $p \leq 0.001$.

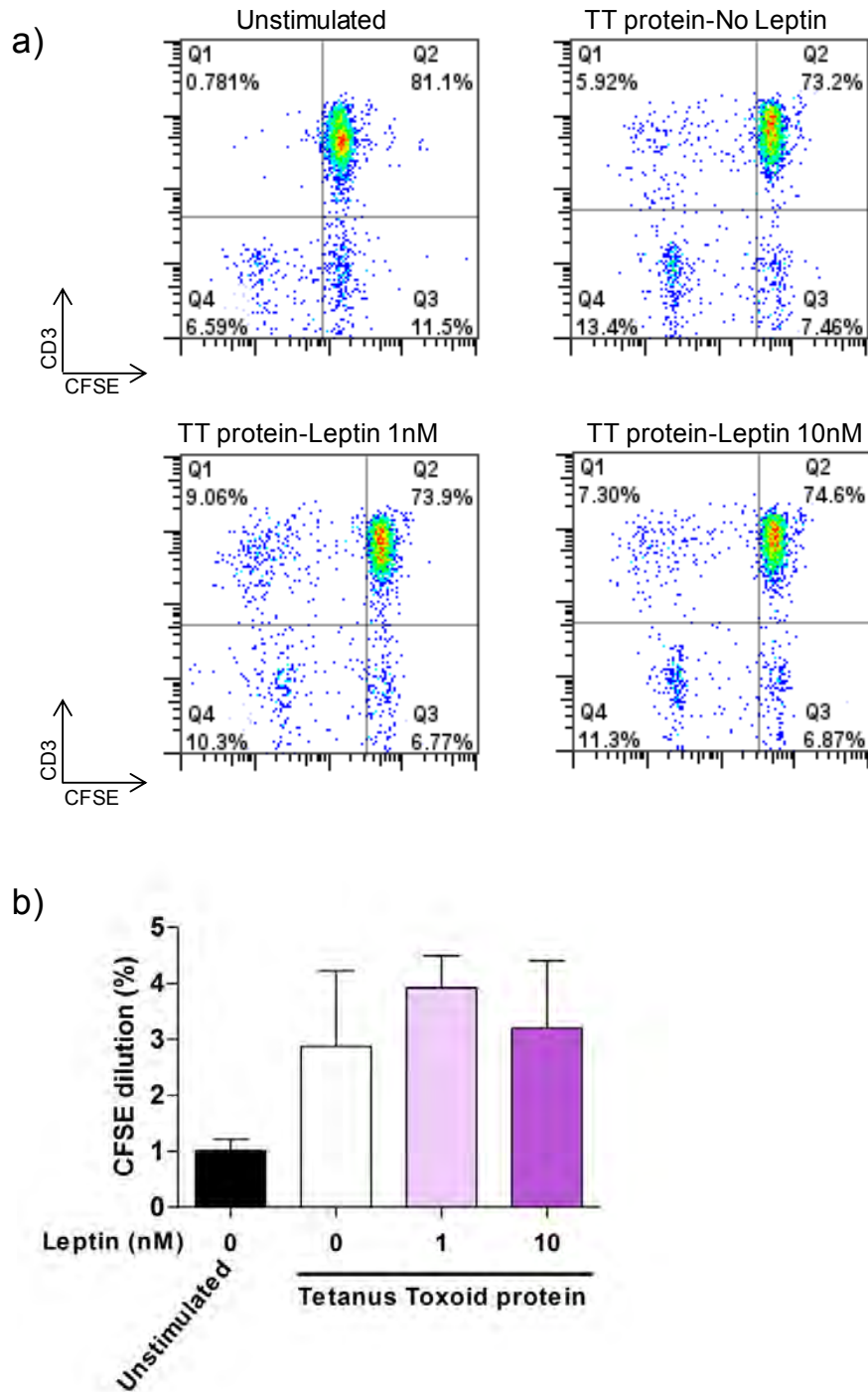


Figure 4-5: Leptin has no effect on Tetanus Toxoid protein-induced T cell proliferation

(a) PBMC were isolated from the peripheral blood, labelled with CFSE and stimulated with 5µg/ml TT protein in absence or presence of leptin at 1 or 10nM for seven days. Antigen specific TT protein induced T cell proliferation was measured CFSE dilution. (b) T cell proliferation was slightly induced by TT protein but no significant differences were found with or without leptin. Data are mean±SEM and were analysed using one-way ANOVA and Bonferroni's multiple comparisons post-test. Pool of at least three experiments.

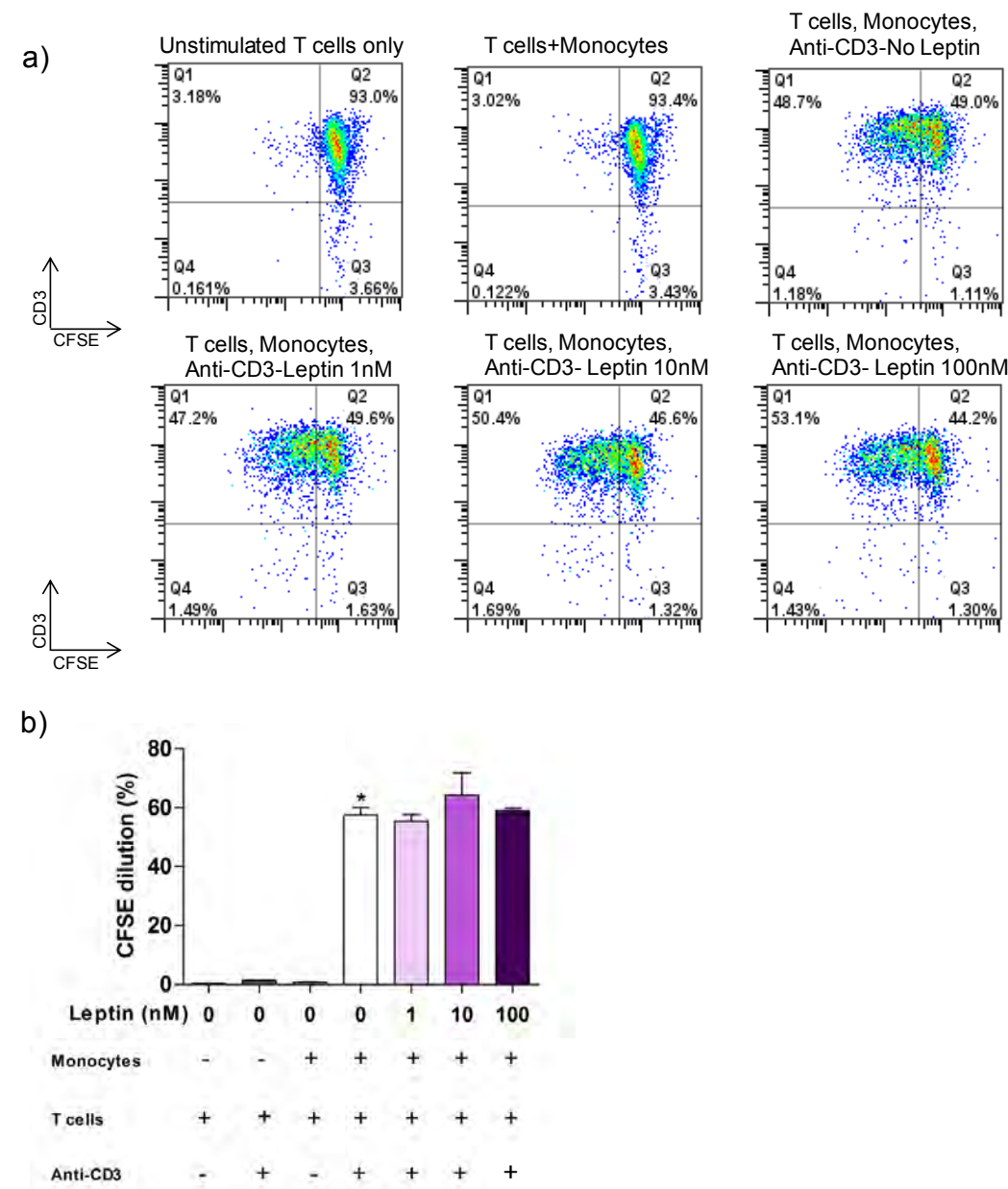


Figure 4-6: Leptin has no effect on anti-CD3 and monocytes-induced T cell proliferation
(a) Monocytes and CD4⁺ CD25⁻ T cells were isolated from PBMC. T cells were labelled with CFSE and stimulated with anti-CD3 (0.5µg/ml) and monocytes at 1 to 10 ratio, in absence or presence of leptin at 1 to 100nM for three days. T cell proliferation was measured CFSE dilution. (b) T cell proliferation was significantly induced in presence of both monocytes and anti-CD3 but no significant differences were found with leptin. Data are mean±SEM and were analysed using one-way ANOVA and Bonferroni's multiple comparisons post-test. Pool of at least three experiments. *p≤0.05.

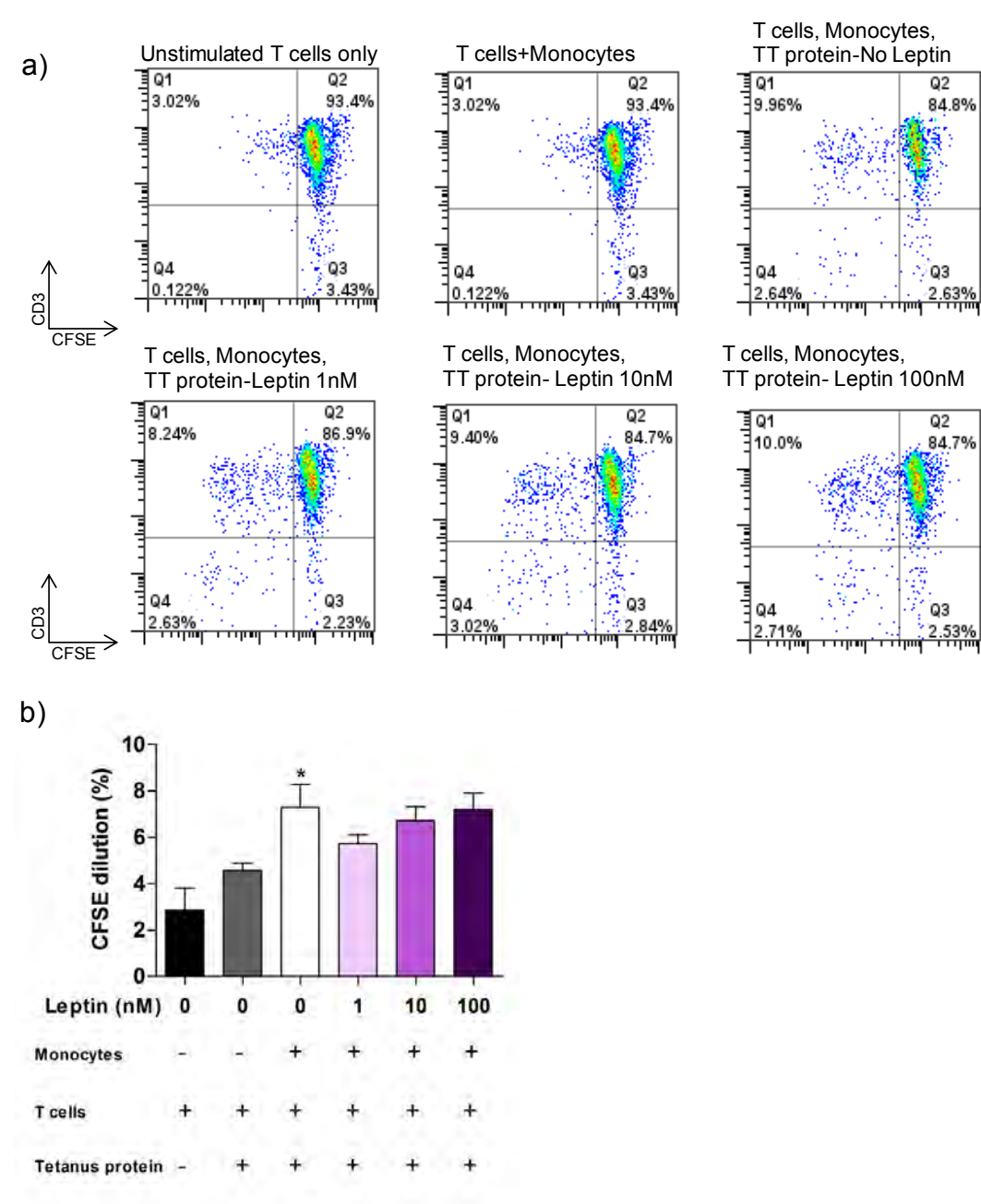


Figure 4-7: Leptin has no effect on TT protein and monocytes-induced T cell proliferation

(a) Monocytes and CD4⁺ CD25⁻ T cells were isolated from PBMC. T cells were labelled with CFSE and stimulated with TT protein (3µg/ml) and monocytes at 1 to 10 ratio, in absence or presence of leptin at 1 to 100nM for three days. T cell proliferation was measured by CFSE dilution. (b) T cell proliferation was significantly induced in presence of both monocytes and TT protein but no significant differences were found with leptin. Data are mean±SEM and were analysed using one-way ANOVA and Bonferroni's multiple comparisons post-test. Pool of at least three experiments. *p≤0.05.

2.2. Leptin has no effect on T cell cytokines production

Pro-inflammatory cytokines, such as IFN- γ , IL-2, IL-21, IL17 and TNF- α , are involved in the pathogenesis of T1D. Given the well-characterized pro-inflammatory effect of leptin in immunity, we wished to characterise the effect of leptin on cytokine production by T cell.

CD4⁺CD25⁻ T cells were stimulated with CD3/CD28 Dynabeads in serum-free media for five days in presence of leptin at 0; 0.1; 1; 10 and 100nM. At day 5, cells were re-stimulated with PMA and protein secretion was stopped with Brefeldin A. Then, cells were fixed and stained for intracellular cytokines.

The addition of physiological or supra-physiological amounts of leptin did not influence the production of either pro- or anti-inflammatory cytokines such as IFN- γ , IL-17, IL-2, IL-21 and IL-10 (**Figure 4-8, 9 and 10**)

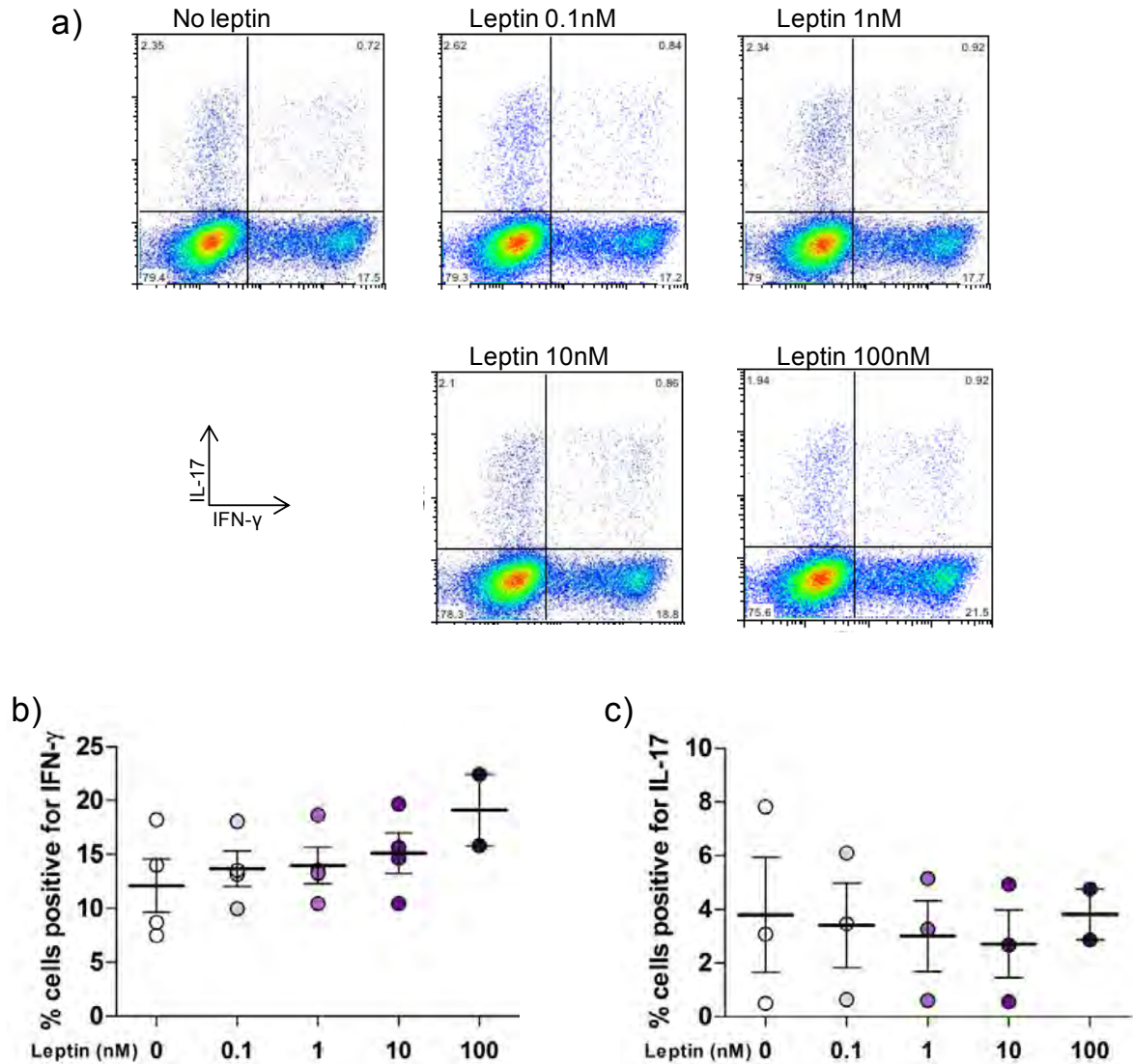


Figure 4-8: Leptin has no significant effect on IFN- γ and IL-17 production

(a) CD4⁺CD25⁻ T cells were stimulated with CD3/CD28 beads (8:1) for 6 days. Cells were re-stimulated with PMA/Ionomycin over night before blockade of protein secretion with Brefeldin A. Leptin was added in the cultures at 0, 0.1, 1, 10 and 100nM. IFN- γ and IL-17 production was measured by intracellular staining. Pool of two experiments realised in duplicate for (b) IFN- γ and (c) IL-17. Some conditions have fewer values because of cell death and lack of staining. Data were analysed using one-way ANOVA and Dunnett's multiple comparisons post-test. No significant differences were found.

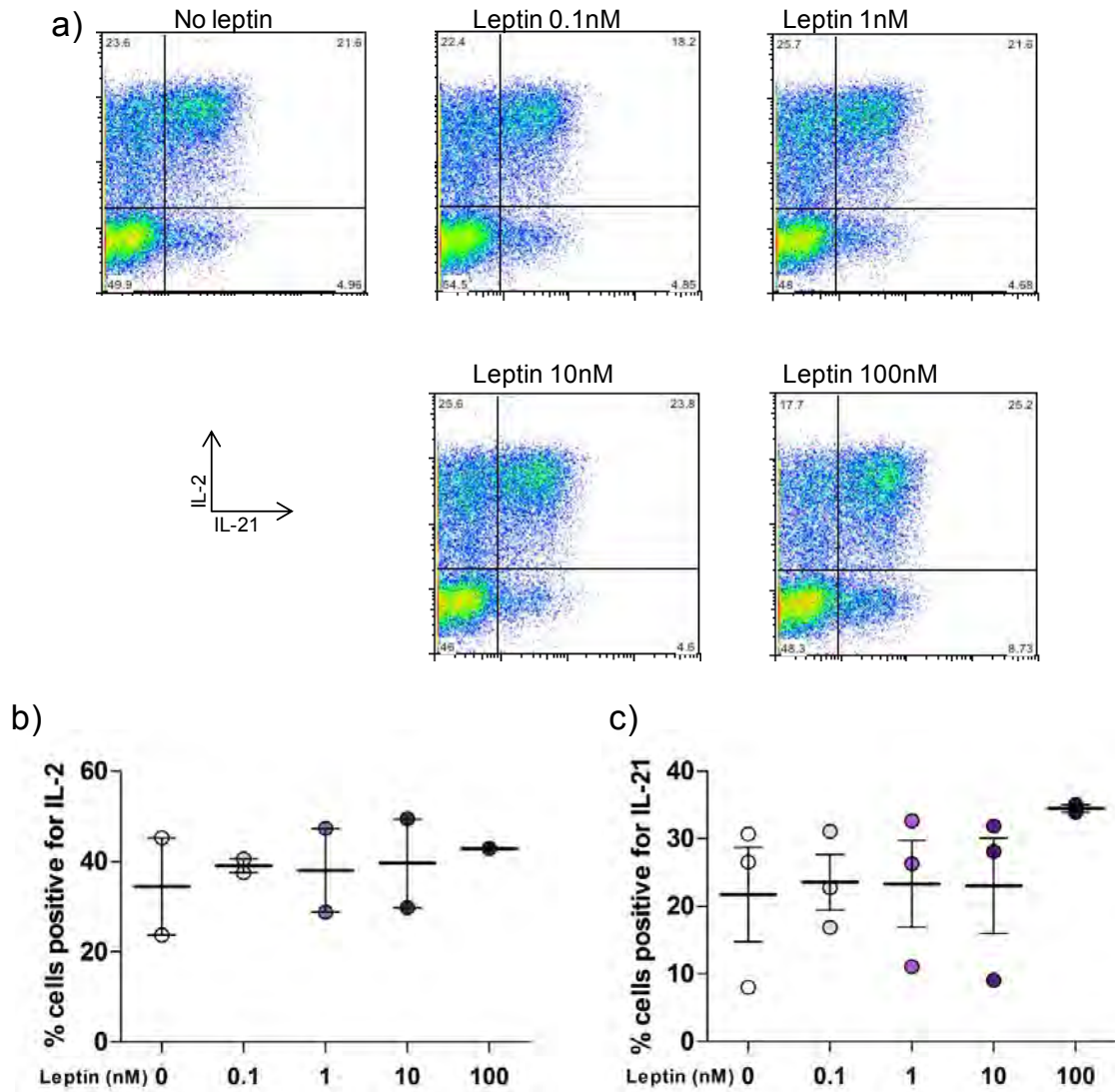


Figure 4-9: Leptin has no significant effect on IL-2 and IL-21 production

(a) $CD4^+CD25^-$ T cells were stimulated with CD3/CD28 beads (8:1) for 6 days. Cells were re-stimulated with PMA/Ionomycin over night before blockade of protein secretion with Brefeldin A. Leptin was added in the cultures at 0, 0.1, 1, 10 and 100nM. IL-2 and IL-21 production was measured by intracellular staining. Pool of two experiments realised in duplicate for (b) IL-2 and (c) IL-21. Some conditions have fewer values because of cell death and lack of staining. Data were analysed using one-way ANOVA and Dunnett's multiple comparisons post-test. No significant differences were found.

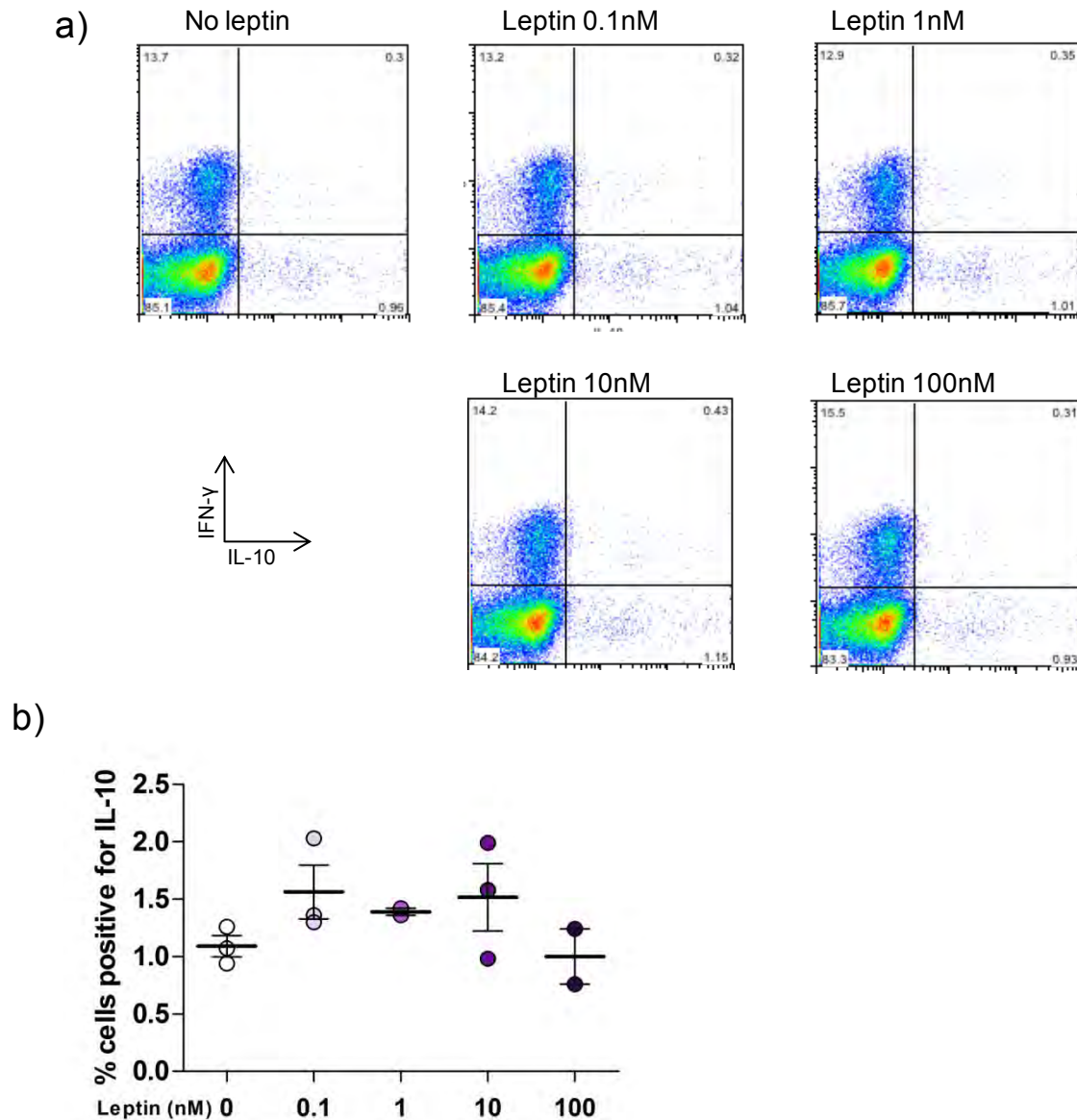


Figure 4-10: Leptin has no significant effect on IL-10 production

(a) $CD4^+CD25^-$ T cells were stimulated with CD3/CD28 beads (8:1) for 6 days. Cells were re-stimulated with PMA/Ionomycin over night before blockade of protein secretion with Brefeldin A. Leptin was added in the cultures at 0, 0.1, 1, 10 and 100nM. IL-10 production was measured by intracellular staining. (b) Pool of two experiments realised in duplicate for IL-10. Some conditions have fewer values because of cell death and lack of staining. Data were analysed using one-way ANOVA and Dunnett's multiple comparisons post-test. No significant differences were found.

2.3. Leptin has no reproducible effect on co-stimulatory molecule expression by DCs

Here, we wished to examine the effect of leptin on the process of DCs differentiation. DCs are routinely differentiated from monocytes from the peripheral blood in presence of IL-4 and GM-CSF in medium containing 10% bovine serum. Because bovine serum contains leptin with the potential to cross react with the human leptin receptor, we needed to develop a serum-free system in which DCs would be efficiently differentiated. CellGroDC serum-free media (CellGenix) has been previously shown to be the optimal medium for the generation of DC (Napoletano *et al.*, 2007).

Using CellGroDC serum-free media, we were able to differentiate cells with phenotypic characteristics of DCs (**Figure 4-11**). In brief, magnetic sorted monocytes showing classic phenotypic markers (CD14⁺⁺⁺, CD11c⁺⁺, HLA-DR⁺⁺, CD86⁺, CD1a⁻, CD80⁻, CD83⁻ and DC-SIGN⁻, **Figure 4-11a**) were differentiated into immature DCs (imDCs) in serum-free conditions. After 5 days, the cells showed a decreased expression of CD14 and an increase in the classical DCs markers (DC-SIGN⁺⁺⁺, CD1a⁺⁺⁺, HLA-DR⁺⁺, CD86⁺, CD80⁺ and CD83⁺, **Figure 4-11b**). This phenotype was similar to the one of DCs generated in serum containing medium (**Figure 4-11c**). The only difference regularly found between serum and serum-free generated DCs, was a lower frequency of CD1a⁺ DCs in serum-free conditions. Finally, these DCs were used to stimulate CD4⁺CD25⁻ T cells using anti-CD3 stimulator and no differences were found in their capacity to co-stimulate T cells (**Figure 4-11d**).

We then sought to investigate the effect on leptin on imDCs phenotype and function. In this assay, leptin was added at a concentration of 10nM during monocytes to DCs differentiation. ImDCs were harvested after 5 days and classic DCs markers were analysed by flow cytometry. Live cells were identified by gating out PI positive cells and on the forward/side scatter profile (**Figure 4-12a**). Normally, in serum conditions, all DCs express CD1a. Because, not all our cells express CD1a in serum-free conditions, we gated on CD1a⁺

cells to ensure we looked at fully differentiated DCs and analysed all markers expression on this subset. Under these conditions, we found that presence of leptin during differentiation induced a near significant increase in the frequency of CD86 positives imDCs (**Figure 4-12-a histograms and b**). A trend was also observed for CD86 surface expression in presence of leptin (**Figure 4-12c**).

We then aimed to test the capacity of the DCs generated in presence of leptin, to stimulate T cells. ImDCs were then co-cultured with CD4⁺CD25⁻CFSE⁺ labelled T cells in serum-free media and stimulated with anti-CD3 antibody for three days. T cells co-activated with DCs differentiated in presence of leptin exhibited significantly higher proliferation compared to T cells stimulated by DCs generated without leptin (**Figure 4-13**).

We also looked at the expression of other DCs markers such as CD80, DC-SIGN and HLA-DR in presence or absence of leptin at 10nM (**Figure 4-14a, b**). Leptin did not affect the expression of these markers on DCs suggesting that the effect is specific to CD86 (**Figure 4-14c**).

We then proceeded to repeat these experiments with a new batch of leptin from the same source. Despite a number of experiments, the results could not be reproduced (**Figure 4-15**).

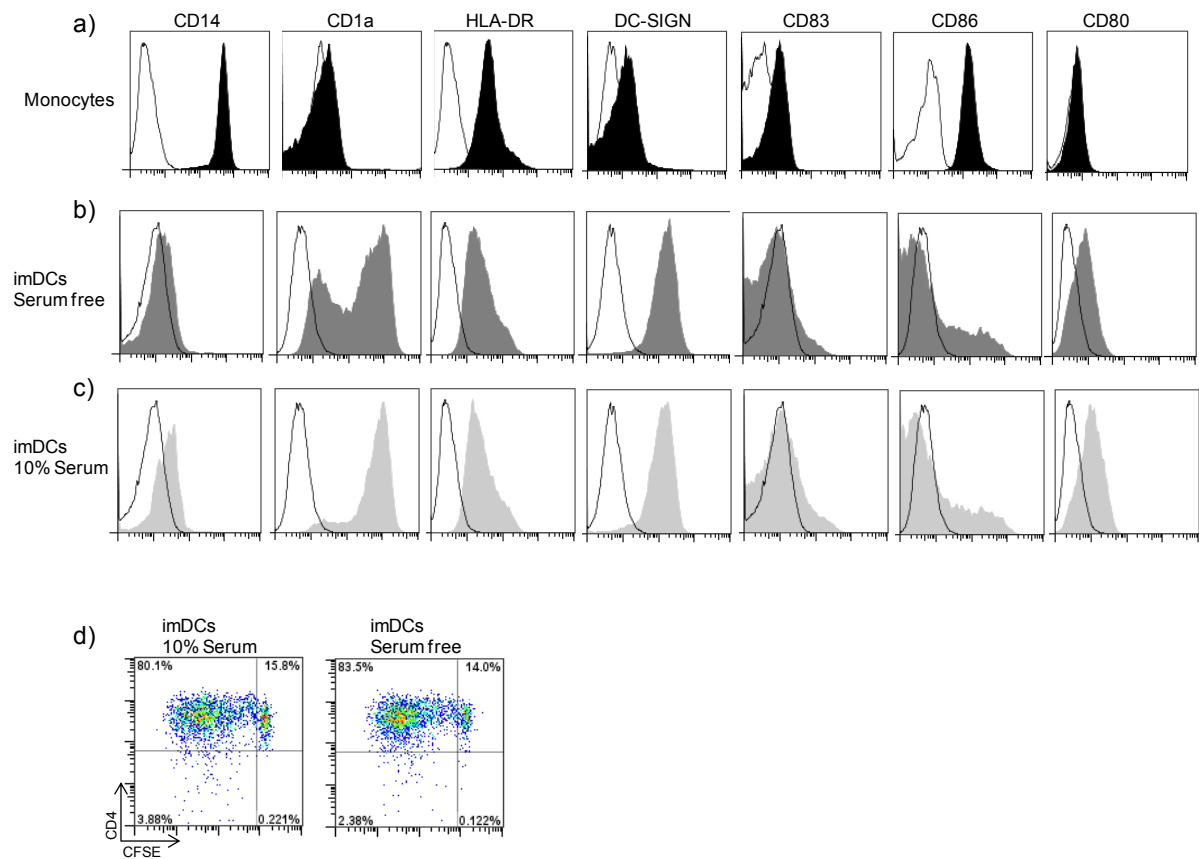


Figure 4-11: Phenotype of DCs generated in serum-free media

(a) Cell surface marker expression was examined on monocytes and (b) imDCs generated in serum-free or (c) 10% of bovine serum conditions using flow cytometry. Other than a decrease in CD1a, there were no differences observed in the phenotype of serum and serum-free generated imDCs. (d) DCs were generated in 10% serum or serum-free media and co-cultured with CFSE labelled CD4⁺CD25⁻ T cells. Proliferation was analysed by CFSE dilution quantification. Data are representative of three independent experiments.

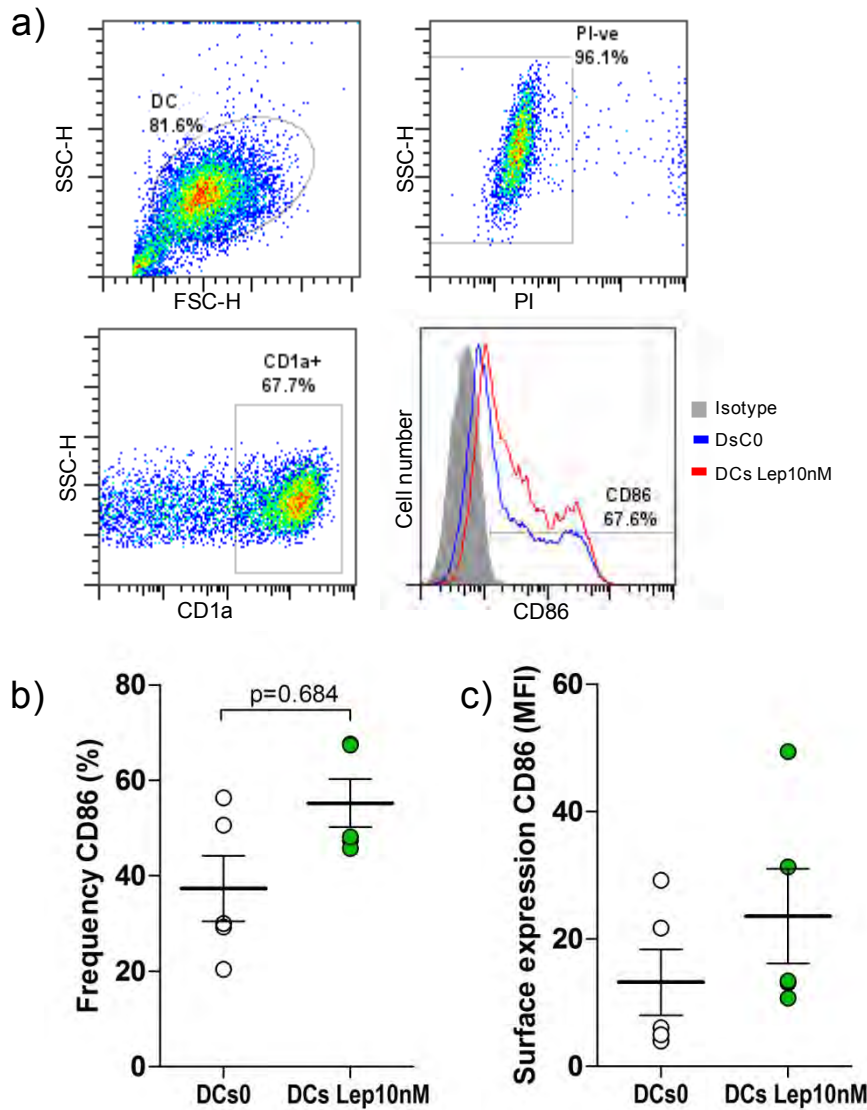


Figure 4-12: Leptin induces a near significant increase of CD86 expression on DCs

(a) Cell surface marker expression was examined on imDCs generated in serum-free with or without leptin using flow cytometry. DCs were gated on their forward/side scatter profile and live cells selected with PI negative staining. CD86 expression was measured on CD1a⁺ DCs. (b) Frequency of CD86 is not significantly increased on CD1a⁺ DCs in the presence of leptin at 10nM and similarly when considering (c) CD86 surface expression (MFI). Pool of at least four experiments Data are mean±SEM and were analysed using t-test.

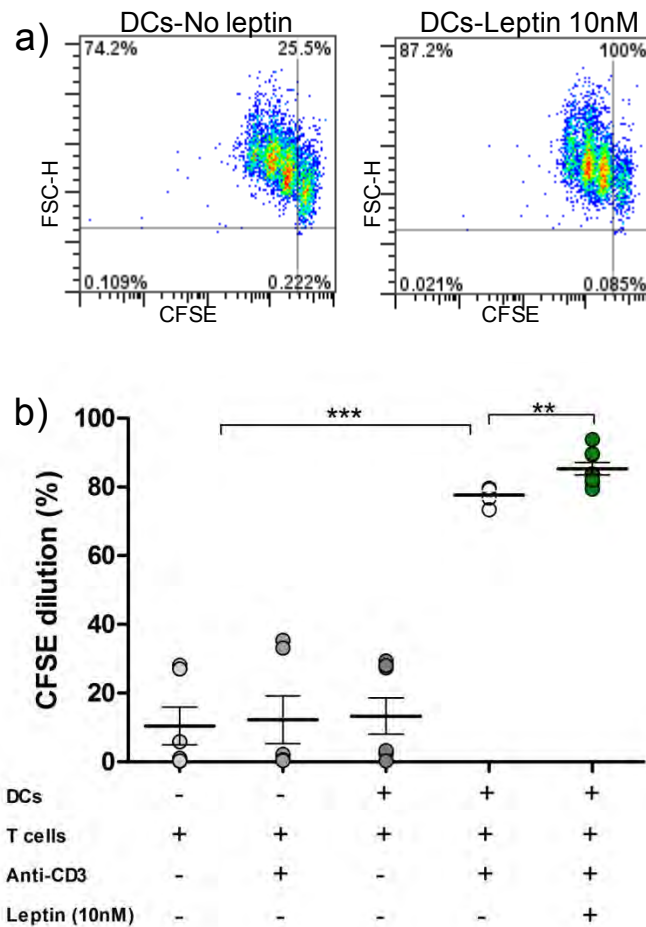


Figure 4-13: DCs generated in presence of leptin have higher T cell stimulatory capacity
 (a) ImDCs generated in serum-free medium with or without leptin were co-cultured with CFSE labelled $CD4^+CD25^-$ T cells. Anti-CD3 was used to stimulate T cells and T cell proliferation was measured by CFSE dilution. (b) T cells were gated on their forward/side scatter profile and live cells were selected with PI negative staining. DCs treated with leptin have significantly higher capacity to stimulate T cells in presence of anti-CD3. Pool of at least three experiments carried out in triplicates. Data were analysed using t-test. ** $p \leq 0.01$, *** $p \leq 0.001$.

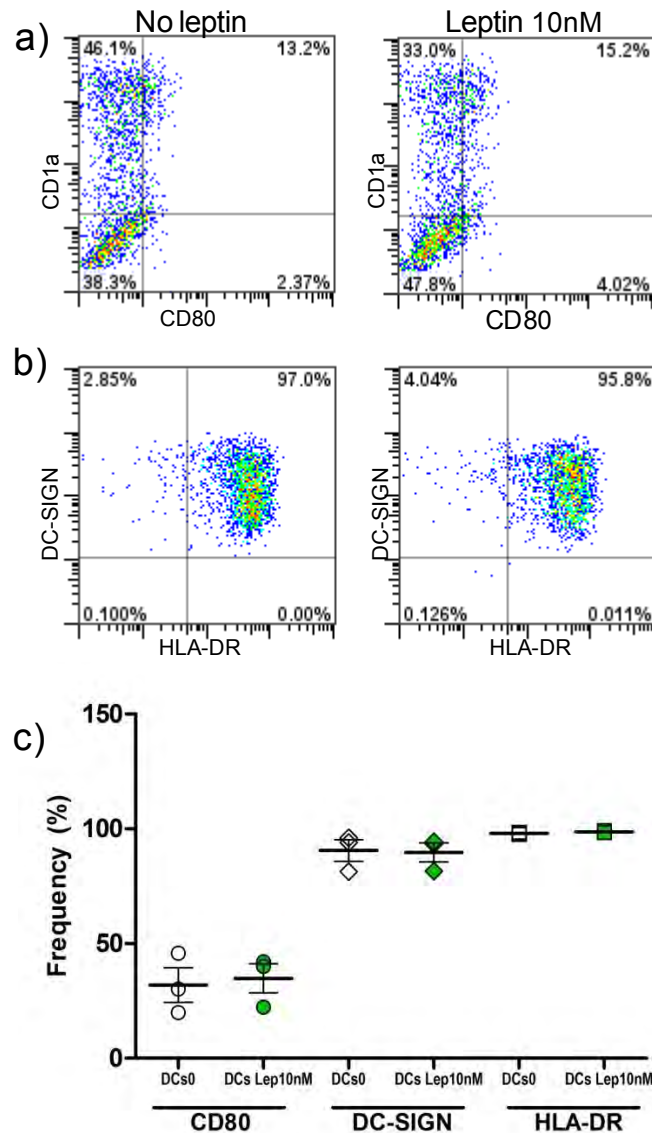


Figure 4-14: Leptin does not affect other imDCs markers

(a) Cell surface marker expression was examined on imDCs generated in serum-free with or without leptin using flow cytometry. DCs were gated on their forward/side scatter profile and live cells were selected with PI negative staining. CD1a⁺ DCs were gated and CD80 (a), DC-SIGN and HLA-DR (b) expression were measured. (b) Leptin did not induce any difference in the frequency of CD1a⁺CD80⁺, DC-SIGN⁺ or HLA-DR⁺ DCs. Pool of at least three experiments (except HLA-DR, N=1). Data were analysed using one-way ANOVA and Bonferroni's multiple comparisons post-test.

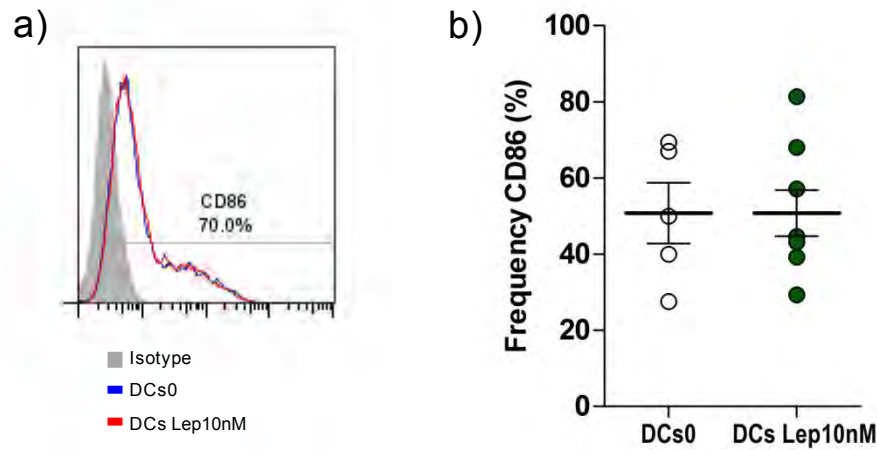


Figure 4-15: Leptin effect on CD86 expression is batch-dependent

(a) Cell surface marker expression was examined using flow cytometry on imDCs generated in serum-free media in the presence of a new batch of leptin. DCs were gated on their forward/side scatter profile and live cells were selected with PI negative staining. CD1a⁺ DCs were gated and CD86 expression was measured. (b) This batch of leptin did not induce a difference in the frequency of CD1a⁺CD86⁺ DCs. Pool of at least three independent experiments. Data were analysed using t-test.

2.4. Leptin has no effect on antigen uptake by APCs

We then sought to further investigate the effect of leptin on the function of DCs by measuring their capacity to take up antigen. Magnetic sorted monocytes were differentiated into imDCs in serum-free in presence of leptin. Dextran-FITC was added at 0.1mg/ml to DCs for 7 minutes at either 4°C or 37°C. These differences allowed us to quantify by flow cytometry, the amount of Dextran-FITC that is internalised (37°C) and which is bound to the cell surface (4°C). Dextran-FITC uptake is then quantified by subtracting the MFI or percentage of Dextran-FITC positive cells at 4°C to 37°C.

Under these conditions, DCs efficiently internalised Dextran-FITC at 37°C but not at 4°C (**Figure 4-16a**). The gate to measure Dextran-FITC uptake was set up on the control DCs without the antigen. Dextran-FITC uptake is unchanged in DCs differentiated in the presence of leptin (**Figure 4-16b**). The cell surface binding of Dextran-FITC measured at 4°C on DCs was also confirmed to be similar in the presence of leptin.

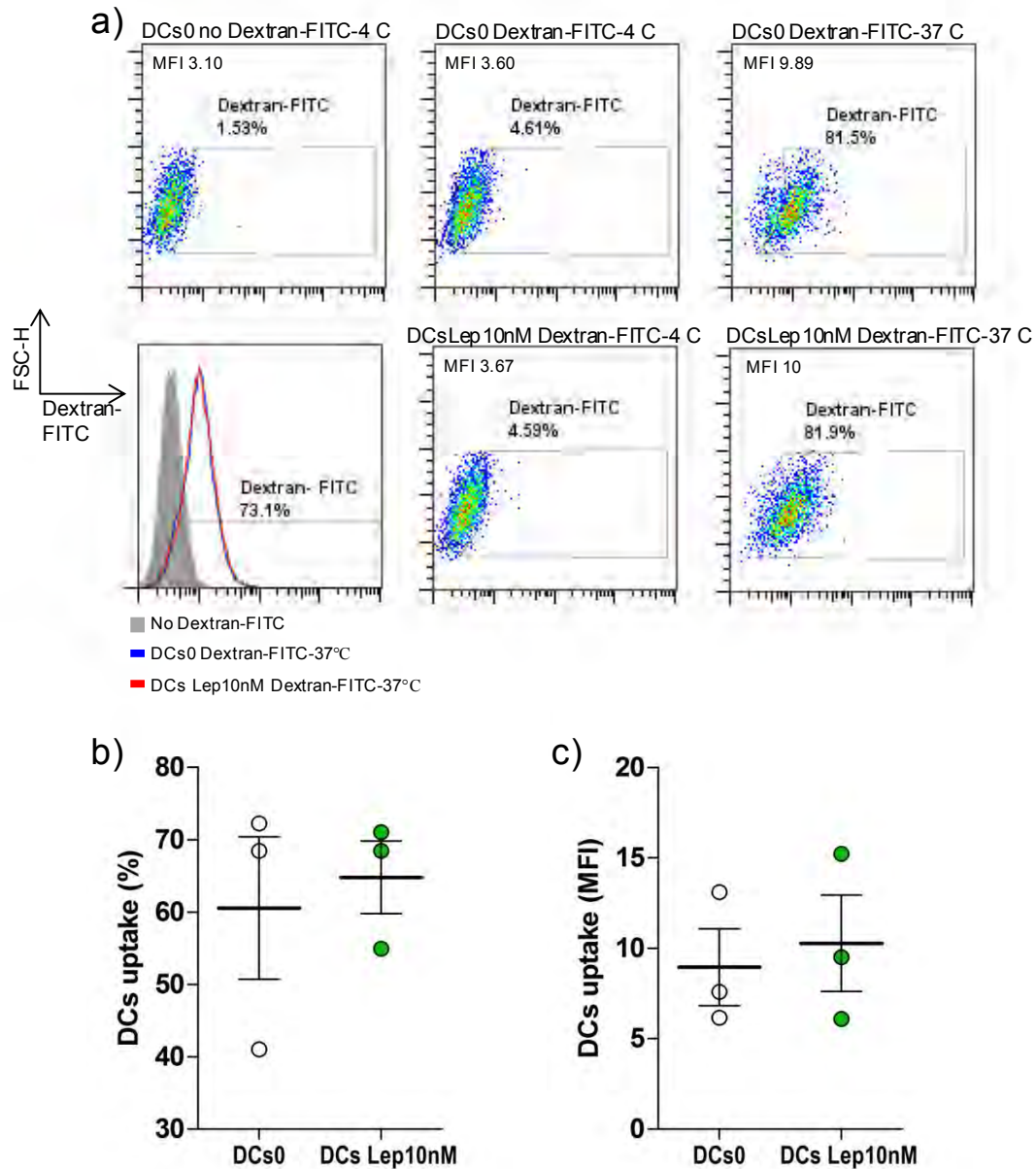


Figure 4-16: Leptin has no effect on antigen uptake by DCs

(a) DCs were differentiated in presence of 10nM leptin. Dextran-FITC was added for 7 minutes at 37°C and at 4°C, washed two times using PBS at 4°C and uptake was analysed by flow cytometry. Dot plots representative of Dextran-FITC uptake at 4°C and 37°C. (b) The cell surface binding measured at 4°C was subtracted from the percentage of DF positive cells at 37°C and (c) the same for the MFI. Cell surface binding and uptake were similar between DCs untreated or treated with leptin. Pool of at least three experiments. Data were analysed using t-test.

2.5. Optimisation of lymphocytes transmigration across HUVEC in low serum conditions

Measurement of lymphocyte adhesion and transmigration was previously conducted in media containing 20% of FCS (McGettrick *et al.*, 2009). In these conditions, adhesion and transmigration of lymphocytes across HUVEC treated with TNF- α and IFN- γ for 24 hours was observed. Because leptin and adiponectin are present in bovine serum and are biologically cross-reactive with human cells, it was thought expedient to set up the assay in low serum media to remove the confounding actions of bovine adipokines (Wang *et al.*, 2004). When we compared the adhesion of migration in low serum conditions (2%) or our normal 20% serum conditions, total adhesion, firm adhesion on the top of the endothelium and transmigration of lymphocytes across the endothelium were not significantly affected (**Figure 4-17**).

2.1. Leptin has limited effect on lymphocyte transmigration

Next, we investigated whether leptin could modulate lymphocyte transmigration. We know that PBL express LEPR, but expression has not been investigated in HUVEC. In order to determine through which cell type leptin was mediating its effects we measured LEPR on HUVEC. LEPR was highly expressed on HUVEC when assessed by flow cytometry; indeed levels appeared much higher than on PBMC (**Figure 4-18**).

To determine whether leptin was capable of stimulating endothelial cells so that they could recruit PBL, we treated unstimulated HUVEC with leptin. We observed no difference in total PBL adhesion compared to untreated HUVEC. However, the small proportion of PBL that did adhere to the EC could migrate with significantly greater efficiency when the HUVEC had been stimulated with leptin. The number of surface adherent PBL, which is the reciprocal of the transmigrated fraction showed the appropriate reduction associated with the more efficient transit of lymphocytes across the monolayer (**Figure 4-19**).

We went on to determine whether leptin could regulate PBL recruitment on cytokine stimulated HUVEC. Importantly, stimulation of the HUVEC with cytokines caused a dramatic increase in total adhesion and transmigration of PBL compared to untreated cells (**Figure 4-20**). Under these conditions of cytokine stimulation, we treated either the HUVEC or PBL with 10nM of leptin (**Figure 4-20**). Treatment of HUVEC with leptin did not change the total number of PBL recruited. However, leptin did induce interesting variations in lymphocyte behaviour. Thus, there was a significant decrease of PBL transmigration along with the appropriate increase in the reciprocal measure of firm adhesion (**Figure 4-20**). Treatment of PBL with leptin had the same effect but the magnitude of the inhibitory response was more pronounced.

Finally, we tested whether the treatment of HUVEC with leptin could modulate the expressions of adhesion molecules such as ICAM-1, VCAM-1 and E-selectin. These changes were assayed by flow cytometry using unstimulated, TNF- α /IFN- γ stimulated, and TNF- α /IFN- γ -leptin stimulated HUVEC. Leptin did not induce expression of adhesion receptors on unstimulated HUVEC. Importantly however and as expected, we saw up-regulation of VCAM-1 and ICAM-1 in response to cytokine treatment. E-selectin did not change at this time point (24h), but E-selectin expression is known to peak at approximately 6h and return to baseline within 24h (**Figure 4-21**). The treatment of cytokine stimulated HUVEC with leptin did not result in changes in adhesion receptor expression (**Figure 4-21b**) or surface expression (**Figure 4-21c**).

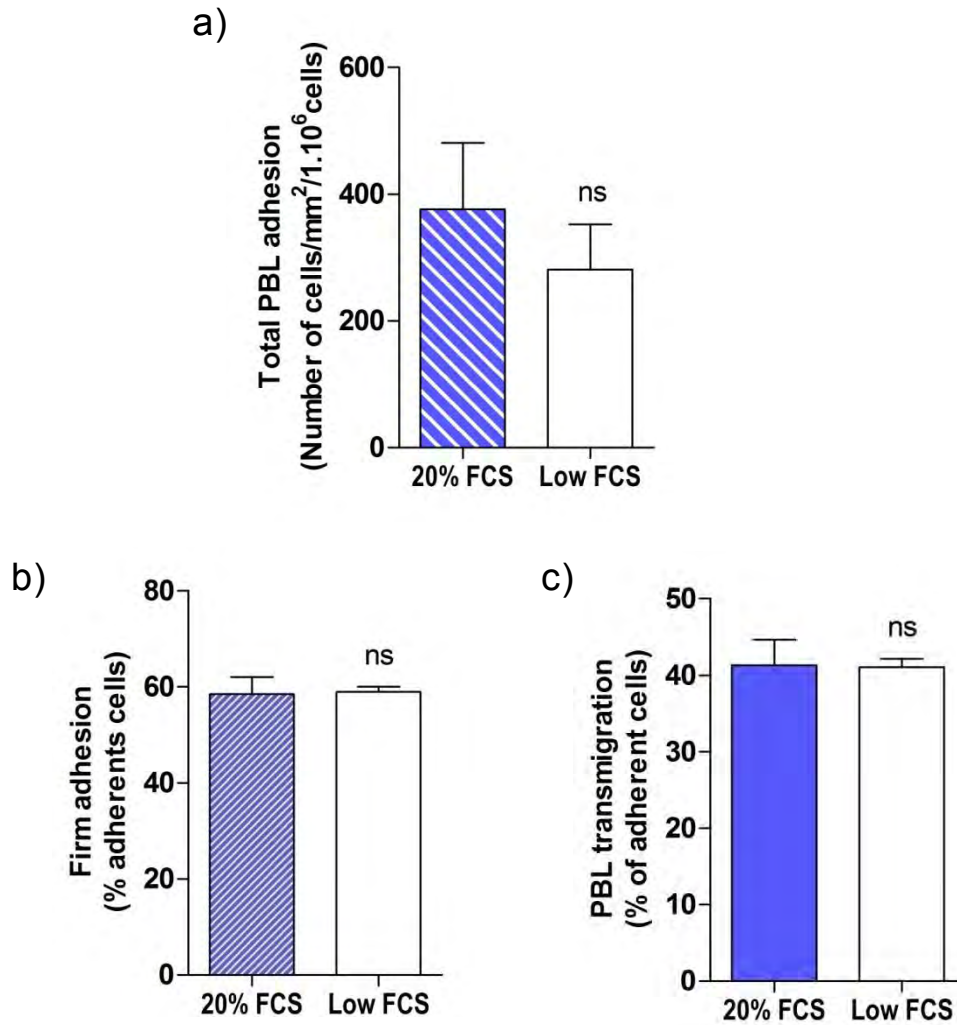


Figure 4-17: The effect of varying the concentration of serum on the adhesion and migration of PBL

HUVEC were grown in 20% FCS complete medium and transferred to a 12 well plate in low serum medium (2%) or left in 20% FCS medium. HUVEC were stimulated with TNF- α and IFN- γ for 24 hours and the level of (a) total adhesion, (b) firm adhesion and (c) transmigration across the endothelium were assessed in static conditions. There was no significant effect on these parameters in low serum conditions. Pool of at least three independent experiments. Data were analysed using t-test. ns=non significant.

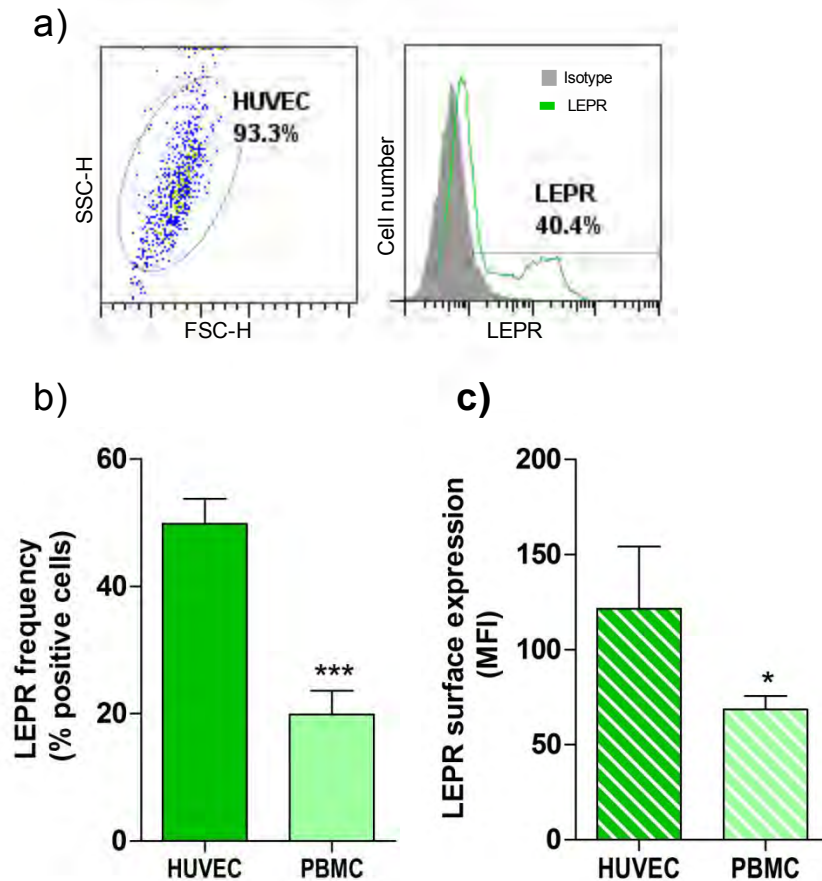


Figure 4-18: LEPR is expressed on HUVEC

(a) The expression of LEPR on HUVEC was determined by flow cytometry. HUVEC were gated on their forward/side scatter profile and LEPR expression (green line) was determined using the isotype (solid grey) as negative control to set the LEPR gate. (b) Frequency of cells positive for LEPR and (c) surface expression of LEPR are higher for HUVEC than PBMC. Data are a pool of at least three independent experiments and were analysed using t-test. * $p \leq 0.05$, *** $p \leq 0.001$.

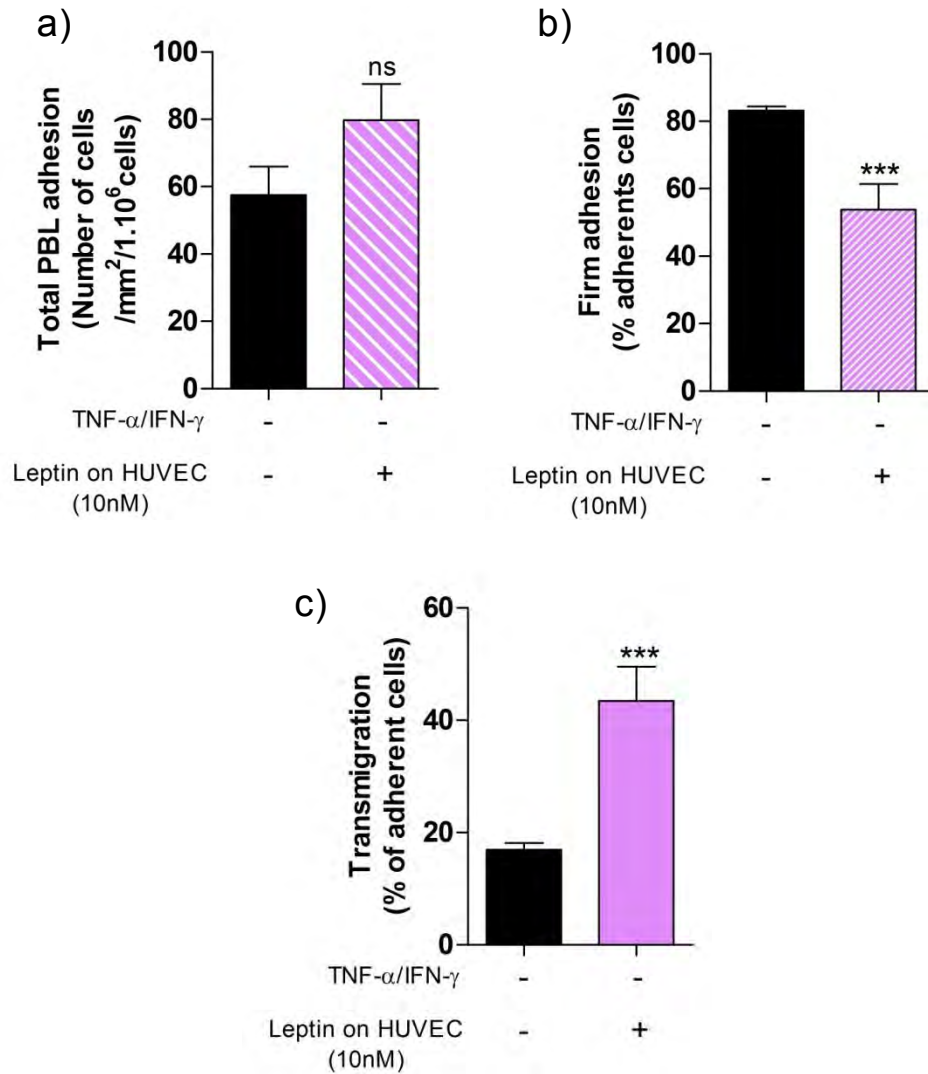


Figure 4-19: Treatment of unstimulated HUVEC with leptin increases PBL transmigration

HUVEC were cultured in low serum media in presence or absence of 10nM leptin for 24 hours. (a) Total adhesion, (b) firm adhesion on the top of the endothelium and (c) transmigration were measured in these conditions. Data are a pool of at least three independent experiments and were analysed using t-test. *** $p \leq 0.001$, ns=non significant.

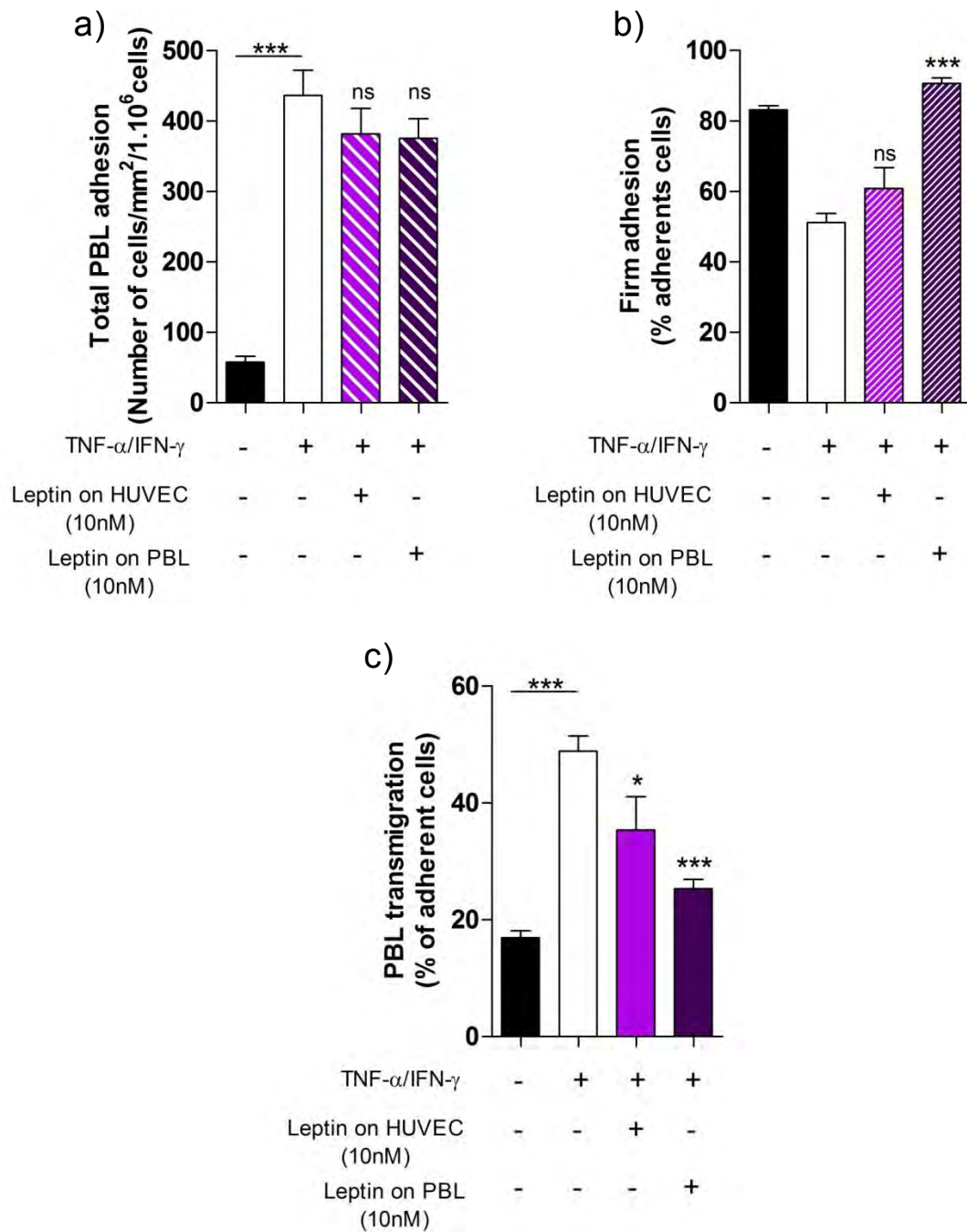


Figure 4-20: Treatment of stimulated HUVEC and PBL with leptin decreases PBL transmigration

Stimulated HUVEC and freshly isolated PBL were cultured in low serum media in presence or absence of 10nM leptin during 24 hours for the HUVEC and 1 hour for the PBL. (a) Total adhesion, (b) firm adhesion on the top of the endothelium and (c) transmigration were measured in these conditions. Data are a pool of at least three independent experiments and were analysed using one-way ANOVA and Bonferroni's multiple comparisons post-test. * $p \leq 0.05$; *** $p \leq 0.001$, ns=non significant.

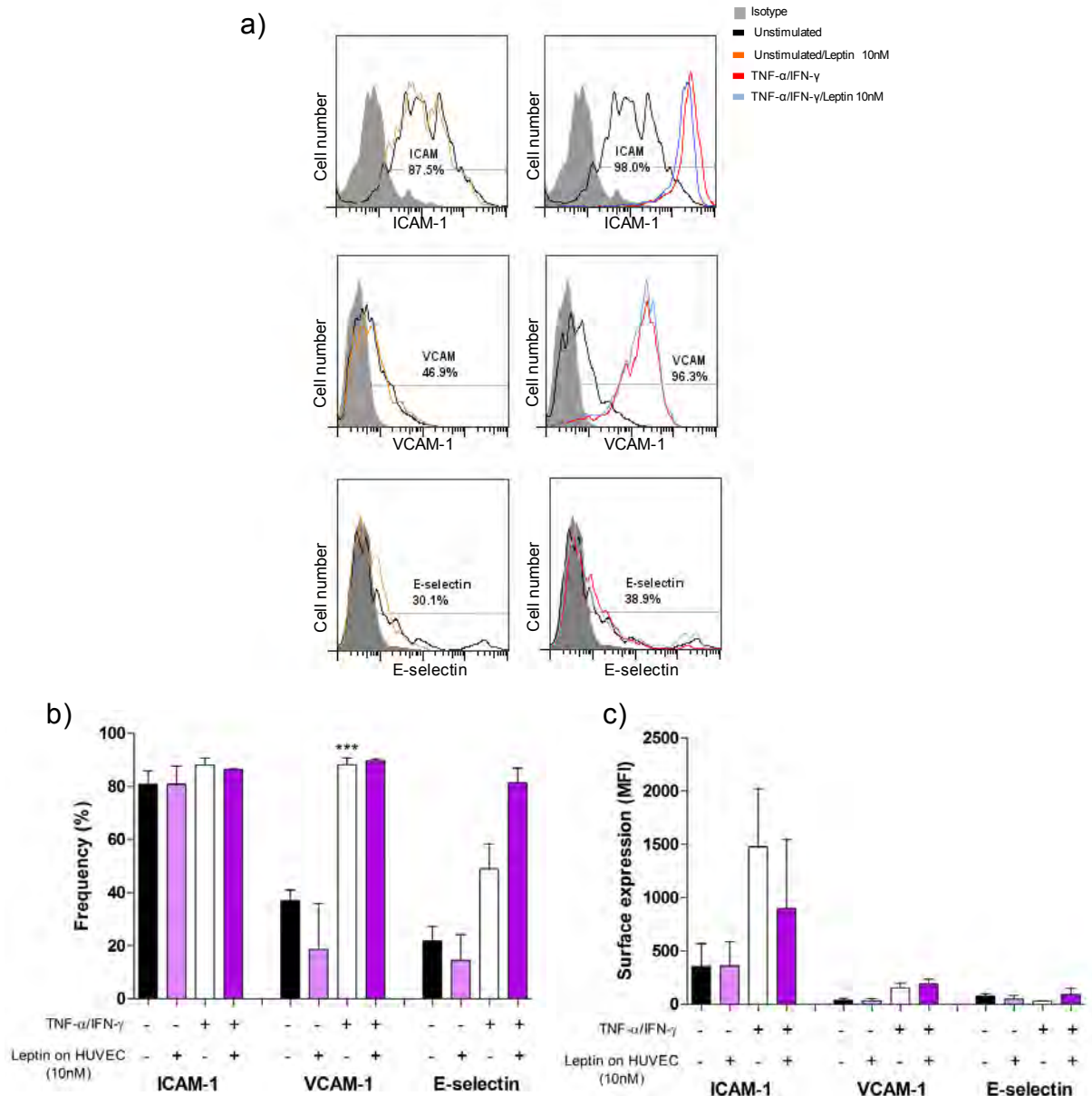


Figure 4-21: Leptin does not affect adhesion molecules expression by HUVEC

(a) ICAM-1, VCAM-1 and E-selectin expression on unstimulated (left panel) and stimulated with TNF- α /IFN- γ for 24 hours (right panel) HUVEC. HUVEC were cultured in absence or presence of leptin at 10nM and adhesion molecule expression was measured by flow cytometry. (b) No significant changes were observed with leptin treatment for ICAM-1, VCAM-1 and E-Selectin frequency and (c) surface expression. Data are a pool of at least two experiments and were analysed using one-way ANOVA and Bonferroni's multiple comparisons post-test. *** $p \leq 0.001$.

3. Discussion

In this chapter, I demonstrate that leptin does not affect T cell proliferation, T cell production of intracellular cytokines, or antigen uptake by DCs. Whilst I was able to demonstrate leptin effect on the *in-vitro* generation of DCs from monocytes; I show that this is batch-dependent. Finally, I demonstrate that leptin is able to promote lymphocyte transmigration across the endothelium in absence of inflammation, but inhibits lymphocyte recruitment in presence of inflammatory signals.

Here, we first aimed to reproduce some of the data available in the literature, which describe the effect of leptin on T cell responses and DCs phenotype and function using serum-free conditions in order to understand the potential functional significance of the differences in LEPR expression in T1D.

However, we found no differences in CD3⁺ T cell proliferation in presence of leptin in whole PBMC or sorted CD4⁺CD25⁻ T cells plus monocytes assays. Measurement of antigen-specific T cell proliferation is critical for our eventual goal of understanding cellular immunity against the pancreatic islet. We did not observe an effect of leptin on antigen-specific T cell proliferation. We then chose to measure cytokine expression using intracellular staining. This technique was preferred instead of the classically used ELISA quantification because it allows co-staining with other T cell markers. We found no major differences in the expression of IFN- γ , IL-2, IL-21 and IL-10 in presence of leptin in response to polyclonal activation.

We then developed a system in which monocytes are efficiently differentiated to imDCs in serum-free conditions. The serum-free generated imDC demonstrated expression of the classical phenotypic markers of DC such as CD80, CD86, CD83, HLA-DR and DC-SIGN, at similar levels to DCs generated in presence of serum. This is in agreement with

published observations using the same CellGroDC media (Napoleitano *et al.*, 2007). The only noticeable exception was a slightly lower frequency of CD1a⁺ cells in serum-free conditions. Although, CD1a is a classic marker of DCs, not all DCs that can perform as APCs have CD1a (Gogolak *et al.*, 2007). In addition, we obtained a satisfactory and similar T cell proliferation at a 1:10 DC to T cell ratio using DCs generated in serum-free and serum-containing media. We confirmed with the company (Cellgenix) the absence of leptin, insulin and other adipokines in the CellGrow media under confidential agreement. Therefore, we concluded that this media was optimal to study the effect of leptin on DCs phenotype and function.

When leptin was added during DC differentiation, we observed a slight significant rise in CD86 expression. ImDCs generated in presence of leptin displayed higher co-stimulation capacities. We found no differences in CD80, HLA-DR and DC-SIGN expression with leptin. Unfortunately, we failed to repeat these findings using a new batch of leptin from the same company. We tested a new source of leptin, added leptin at different times and different concentrations, but none of this modification altered CD86 expression. We concluded that the preliminary effect observed was caused by LPS contamination of leptin. Finally, we demonstrated that uptake of antigens by DCs in presence of leptin is not affected.

This work shows no effect of leptin on T cells responses and DC phenotype and function in contrast to published reports. However, we used a variety of techniques to investigate the effect of leptin and all were optimised in serum-free media contrary to previous studies, which used bovine serum in all their assays. We believed that investigating T cell responses in serum-free media would provide more accuracy as this would increase the sensitivity of cells to leptin.

We now believe that the lack of response to leptin may be caused by differential effects on naive and memory T cells. Indeed, studies have now shown that leptin up-regulates naive T cell proliferation but inhibits memory T cell stimulation by anti-CD3 and monocytes

(Lord *et al.*, 2002). Therefore, unless we specifically select memory and naive T cells, we would not be able to pick up differences in whole PBMC or whole CD4⁺ T cells assays as any increase of proliferation would be blunt by any inhibition.

Another potential rationale behind the discrepancies that we observe compared to published results, would be a specific action of leptin on APC. Indeed, some reports show specific stimulation of monocytes but not T cells by leptin (Santos-Alvarez *et al.*, 1999, Zarkesh-Esfahani *et al.*, 2001). In addition, recent evidence has demonstrated that leptin increases cytokine and chemokine production such as IL-1 β , IL-6, IL-12 and TNF- α and reduction of IL-10 by human imDCs *in vitro* (Mattioli *et al.*, 2005 and 2008). These two studies have also determined that leptin increases imDCs motility by inducing actin filament polarisation and trigger higher T cells responses towards a Th1 phenotype. Leptin can also protect imDCs from apoptosis and these DCs are able to enhance strong CD8⁺ T cells responses (Mattioli *et al.*, 2005 and 2008). Others have shown that absence of leptin receptors affects bone marrow derived DCs number and function *in vivo* (Lam *et al.*, 2007). Indeed, *db/db* mice have lower number of DCs because of high rate of apoptosis. In addition, these DCs have reduced co-stimulatory molecules expression and a Th2 cytokine profile that reduced their capacity to stimulate T cells. However, we did not find any modulation of DCs phenotype and function by leptin.

Finally, we investigated the effect of leptin on PBL trans-endothelial migration. These assays were historically developed in medium containing 20% of bovine serum, optimal to HUVEC growth and maintenance. High levels of adipokines can be found in bovine serum and show biological activity (Wang *et al.*, 2004). Therefore, a 20% supplementation of the media results in high levels of contaminating adipokines in the experimental assays. Hence, we optimised both static and flow assay with HUVEC cultured in low-serum medium (2%)

prior to the experiments. In these conditions, we found no difference in the capacity of low-serum-cultured HUVEC to support recruitment, firm adhesion and transmigration compared to the classic method with 20% serum. In this chapter, we therefore worked using this low serum system in both static and flow conditions and this allowed us to study the full effect of the adipokines we considered.

Given the reported pro-inflammatory properties of leptin, we hypothesised that TNF- α /IFN- γ -induced transmigration through endothelium would be increased in the presence of this adipokine. Interestingly, our data on LEPR indicated that leptin might have effects on either lymphocytes or HUVEC, as both have high expression of this receptor. Indeed there was the possibility that it might have differential effects on the different cells. Interestingly, we observed a significant effect of leptin on the transmigration of PBL when HUVEC unstimulated with cytokines were treated with leptin and used as the adhesive substrate. PBL transmigration was significantly increased with a concomitant decrease of firm adhesion. This observation is in agreement with previous studies showing that leptin was important for facilitating the migration of monocytes and macrophages (Gruen *et al.*, 2007). We did not investigate the effect of leptin pre-treatment on PBL in unstimulated HUVEC conditions, although in the light of these observations, this would be worth examining as well. The fact that the EC were unstimulated by cytokines may indicate that leptin plays a role in the basal trafficking of PBL which occurs during immune surveillance, a process that occurs independently of inflammation.

Intriguingly, we found that when an inflammatory stimulus was utilised to stimulate the HUVEC (i.e. IFN- γ /TNF- α), the effects of leptin were “anti-inflammatory”, i.e. transmigration was significantly reduced. Moreover, this effect was evident whether HUVEC or PBL were treated with leptin. The effects of leptin in this assay were not dependent upon

regulation of endothelial cell adhesion receptors, as we did not see any changes in the levels of VCAM-1, ICAM-1 or E-selectin after treatment of HUVEC with this agent. The anti-inflammatory effects of leptin are not unprecedented, as others have shown similar effects on proliferation of T cells when naive T cells were studied (Okamoto *et al.*, 2000; Lord *et al.*, 2002).

We believe that more stringent studies are merited to understand the effects of leptin on lymphocyte trafficking. These experiments could involve manipulation of the cytokine stimulatory regimen, as it is possible that leptin may induce differential responses in HUVEC activated through different routes. Should the pattern of lymphocyte trafficking observed here be confirmed this may indicate a physiological role for leptin in immune surveillance as well as a role in regulating lymphocyte traffic in inflammation. Indeed, under physiological conditions, lymphocytes are recruited into tissues to patrol for antigens, thus allowing immune surveillance. Trafficking is highly increased during inflammation, however, the lymphocyte subsets recruited may not be the same and their effector functions in these two scenarios are likely to be divergent in order to match the needs of process being supported. A good example of this is in the liver where lymphocytes continuously patrol in the absence of inflammation and different populations can be rapidly expanded in response to infection or injury by recruiting lymphocytes from the blood (Lalor *et al.*, 2002). In this work, we show that leptin may be able to regulate both of these aspects of lymphocyte trafficking, promoting basal immune surveillance, while providing a check to the magnitude of the inflammatory response.

- **Conclusions**

I do not find that leptin modulates T cell responses or APC phenotype and function to the degree described in published literature. Ideally, it would have been interesting to further test the effect of leptin on other DCs characteristics such as secretion of cytokines and morphological changes that are crucial to DCs function (Mattioli *et al.*, 2005, Al-Awan *et al.*, 2001). It would have also been relevant to test the rate of apoptosis in presence of leptin as studies have shown that leptin has anti-apoptotic effects (Mattioli *et al.*, 2009). Finally, leptin is able to modulate lymphocyte trafficking depending on the inflammatory status of the endothelium.

5.CHAPTER 5- THE EFFECT OF ADIPONECTIN ON THE TRANS- ENDOTHELIAL MIGRATION OF LYMPHOCYTES

1. Introduction

Lymphocytes play a key role in the development of T1D (Imagawa *et al.*, 1999, Hanafusa *et al.*, 2008). Lymphocytes traffic to the islets through the pancreatic network of capillaries. However, to infiltrate the islets, lymphocytes must first cross the endothelial cells which line the vessels. Whilst immunotherapies have focused on depleting autoreactive cells, blockade of their trans-endothelial migration could also represent a therapeutic target. Emerging evidence of the immunomodulatory role of adipokines imply their therapeutic potential in this regard.

We were therefore interested in how adipokines influence the migration of lymphocytes across inflamed endothelium. Here, we have examined the effect of the adipokine adiponectin.

Adiponectin is the most abundant circulating adipokine, being present at concentrations of 3-30µg/ml in the blood plasma (Arita *et al.*, 1999). In addition, adiponectin has previously been associated with an anti-inflammatory role in the immune system (**Chapter 1-section 3.2.3**). Data generated in our group by Dr Terence Pang have shown that the expression of adiponectin receptors on PBMC from patients with T1D is decreased, while no changes were observed for circulating adiponectin levels. Moreover, adiponectin is associated with inhibition of leukocyte migration (Cao *et al.*, 2009, Ouedraogo *et al.*, 2007). Mice lacking adiponectin exhibit a 2-fold increase of leukocyte rolling and a 5-fold rise in leukocyte adhesion on endothelium in peri-intestinal venules when assessed using intravital microscopy. Adiponectin had similar effects on adhesion of leukocytes in the isolated aortas of adiponectin knock-out mice (Ouedraogo *et al.*, 2007). This study and others have shown that adiponectin may regulate leukocyte migration by altering endothelial expression of VCAM-1 and E-selectin (Ouedraogo *et al.*, 2007; Ouchi *et al.*, 1999; Cao *et al.*, 2009). Whilst these *in vivo* studies using mice indicate a role for adiponectin in the control of leukocyte

migration, no data is available using human cells. Here, we report the first data on the role of adiponectin in regulating the trafficking of human leukocytes. We chose to use HUVEC as a model of endothelium for the reasons mentioned in the previous chapter. Furthermore, other studies have shown the expression of both adiponectin receptors in HUVEC (Hattori *et al.*, 2003; Lee *et al.*, 2008; Zhang *et al.*, 2008; Xu *et al.*, 2010). It is therefore important to test adiponectin in migration assays using human endothelium and primary leukocytes. Here, we report some of the first assays of this type, in work conducted in collaboration with Drs Ed Rainger and Helen McGettrick. In these studies, we used the same conditions of HUVEC culture optimised in the assays with leptin i.e. low serum conditions and stimulation with TNF- α and IFN- γ .

2. Results

2.1. Adiponectin strongly inhibits lymphocytes transmigration

2.1.1. Treatment of HUVEC with adiponectin affects lymphocyte transmigration

First, we aimed to confirm the expression of both adiponectin receptors AR1 and 2 on HUVEC using flow cytometry and qPCR. AR1 and AR2 expressions were found on HUVECs at both protein and mRNA level (**Figure 5-1**). AR2 expression was significantly higher than AR1 at mRNA level.

To determine whether adiponectin could influence lymphocyte migration, we measured lymphocyte recruitment on TNF- α /IFN- γ -stimulated HUVEC. In static conditions, treatment of HUVECs with 15 μ g/ml of adiponectin for 24 hours did not alter total adhesion of PBL (**Figure 5-2a**). However, there was a significant increase of firm adhesion of PBL on the endothelium and a concomitant reduction of PBL transmigration (**Figure 5-2b, c**).

Increasing dose of adiponectin (0.0001 to 15µg/ml) concentrations diminished the effect on PBL transmigration, thus indicating that the effect is dose-dependent (**Figure 5-2d**).

Next, we repeated these experiments in flow conditions, which more closely represent physiological conditions. Similarly to static assays, total adhesion is unchanged and transmigration is reduced on adiponectin-treated HUVEC (**Figure 5-3**). Interestingly, there is a trend to an increase in rolling with adiponectin treatment, suggesting an impairment of either chemokines-induced activation of leukocytes or changes in the counter receptors for leukocyte integrins.

We went on to verify whether the adiponectin inhibition of PBL transmigration was mediated via AR1 and AR2. AMPK is an important intermediate of AR1 and AR2 signalling that mediates the downstream effects of adiponectin binding to these receptors. Therefore, we used a common inhibitor of AMPK, referred as AMPK compound C, in order to block the adiponectin receptor signalling pathway. Whilst in previous experiments, HUVEC were treated with adiponectin for 24 hours, this degree of exposure to the AMPK compound C was cytotoxic. Therefore, the conditions were adjusted to a 30 minutes treatment of cytokine-stimulated HUVEC with the AMPK compound C followed by one-hour treatment with adiponectin. Adiponectin significantly reduced PBL transmigration even after the brief treatment (**Figure 5-4**). Pre-treatment with AMPK compound C restored PBL transmigration to the control levels, indicating that effects of adiponectin on lymphocyte transmigration were mediated via AR1/AR2 receptors.

However, AMPK is involved in numerous functions and signalling cascades (Reviewed in Hardie, 2011), and off-target effects are therefore possible in this experiment. In order to ensure the involvement of the receptors, we proceeded to knock-down AR1 and AR2 expression in HUVEC using siRNA strategies. Transfection of a pool of four siRNA duplexes targeting the receptors led to an average of 95% and 88% knock-down of AR1 and AR2

respectively (**Figure 5-5a**). Co-transfection of both sets of siRNA duplexes slightly reduced the knock-down efficiency to an average of 87% and 72% for AR1 and AR2 respectively. Scramble siRNA duplexes were used as a negative control. After 48 hours of siRNA incorporation, HUVEC were stimulated with TNF- α and IFN- γ in presence or absence of adiponectin at 15 μ g/ml. Surprisingly, knock-down of both receptors at the same time did not reverse the effects of adiponectin (**Figure 5-5b**). We therefore proceeded to knock down each receptor individually. Preliminary data show that the effects of adiponectin are lost in the absence of AR2, indicating that the effects of adiponectin could be mediated by this receptor. However, the data are representative of a single experiment and should be reproduced in order to draw firm conclusions.

Finally, we tested whether the treatment of HUVEC with adiponectin could down-regulate the expression of adhesion molecule and chemokines. These changes were assayed by flow cytometry using unstimulated, TNF- α /IFN- γ stimulated and TNF- α /IFN- γ /adiponectin stimulated HUVEC. Adiponectin did not influence the frequency, surface expression and gene expression of VCAM-1, ICAM-1 or E-selection (**Figure 5-6a, b**). In addition, we observed no difference in gene expression of the IFN- γ -inducible chemokines in presence of adiponectin (CXCL9-11) (**Figure 5-6c**).

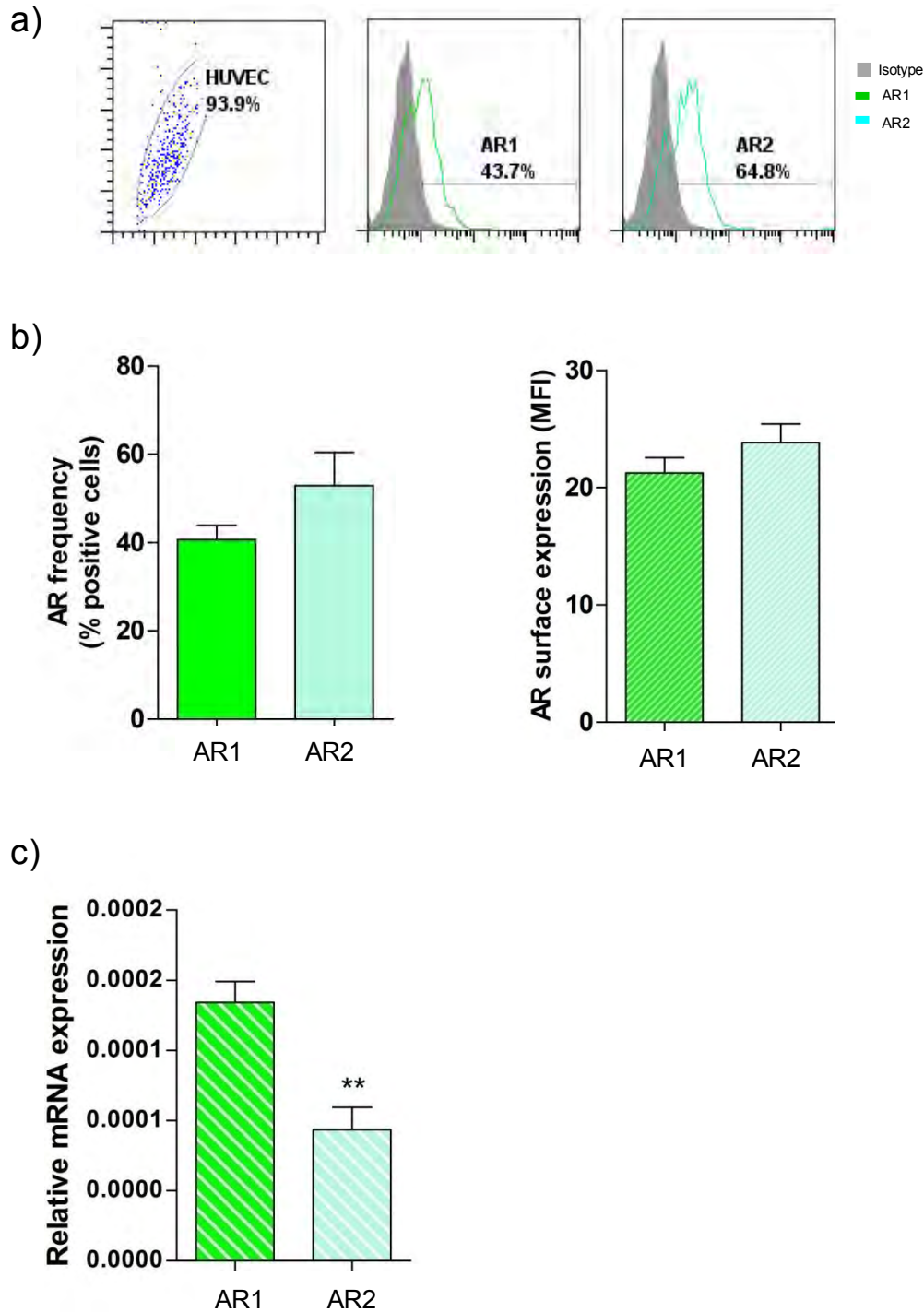


Figure 5-1: ARs are expressed on HUVEC

The expression of ARs on HUVEC was determined by flow cytometry and qPCR. (a) HUVEC were gated on their forward/side scatter profile and AR1 (green line) and AR2 (blue line) expression were determined using the isotype (solid grey) as negative control to set the AR positive gate. (b) Number of cells positive for AR1 and AR2 and their surface expression are similar on HUVEC. (c) AR1 and AR2 gene expression was quantified using qPCR. Data are a pool of at least three experiments and were analysed by t-test. ** $p \leq 0.01$.

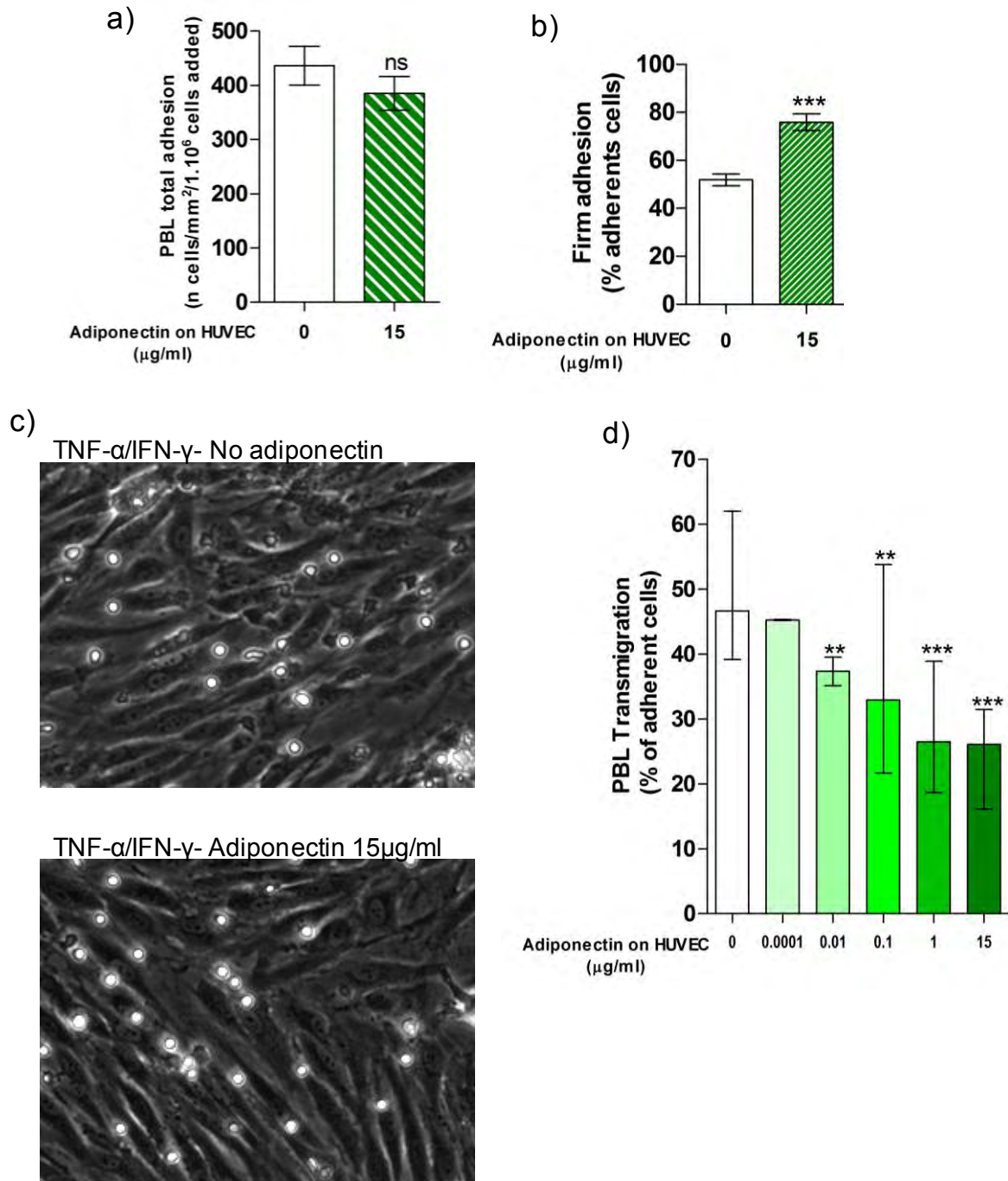


Figure 5-2: Treatment of HUVEC with adiponectin reduces PBL transmigration in static conditions

HUVEC were cultured in low serum medium and stimulated with TNF-α/IFN-γ for 24 hours in absence or presence of increasing dose of adiponectin. (a) PBL adhesion was not affected by adiponectin and (b) firm adhesion is increased. (c) PBL transmigration was significantly reduced from 43% (top) to 17% (bottom) with adiponectin treatment at 15 μg/ml and (d) this effect is dose dependent. Data are a pool of at least three experiments and were analysed using t-test and one-way ANOVA and Dunnett's multiple comparisons post-test. **p ≤ 0.01, ***p ≤ 0.001; ns = non-significant.

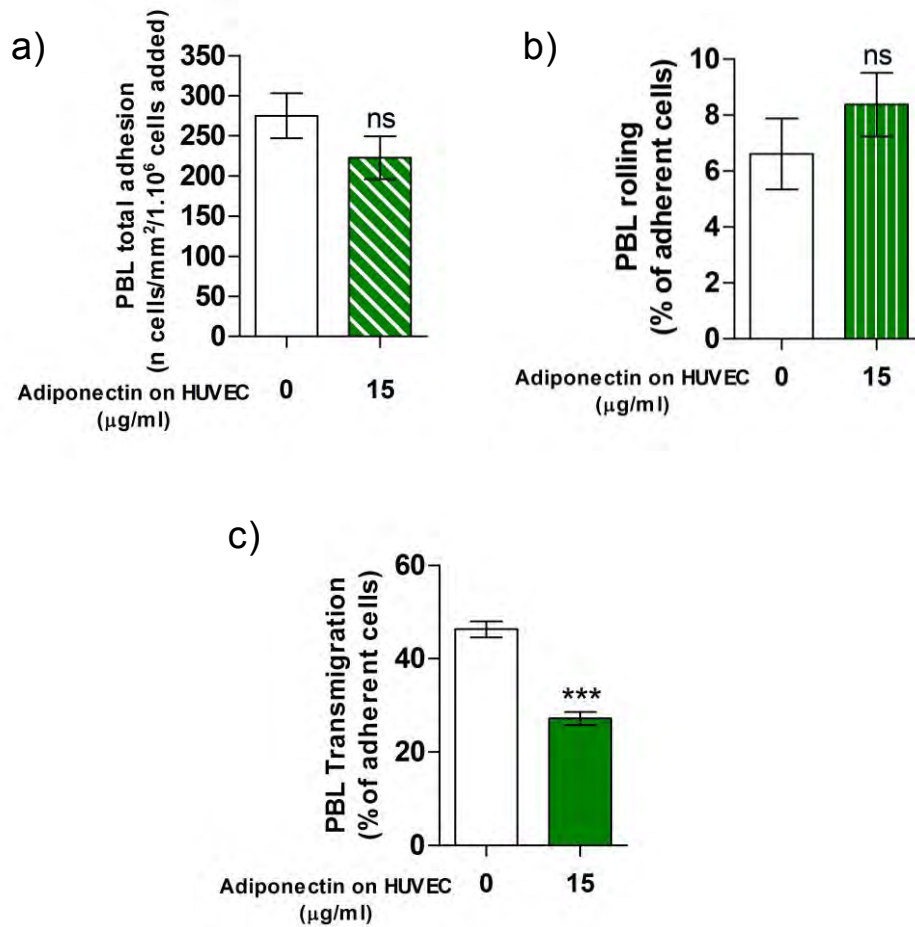


Figure 5-3: Treatment of HUVEC with adiponectin reduces PBL transmigration in flow conditions

HUVEC were cultured in low serum medium in Ibidi slides and stimulated with TNF- α /IFN- γ for 24 hours in absence or presence of adiponectin at 15 μ g/ml. (a) Total PBL adhesion was unchanged and (b) there is a trend to increased rolling. (c) PBL transmigration was reduced with adiponectin treatment of HUVEC. Data are a pool of three experiments realised in duplicates and were analysed using t-test. ***p \leq 0.001.

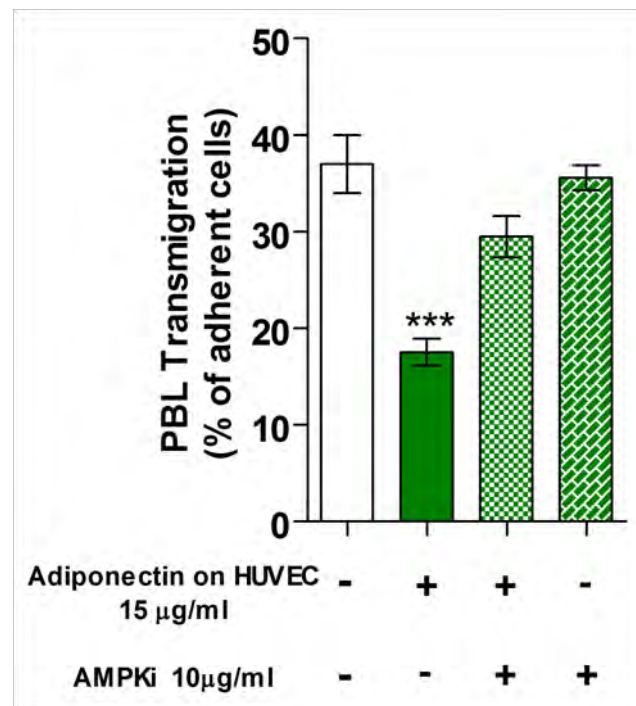


Figure 5-4: AMPK inhibition restores normal level of transmigration

HUVEC were cultured in low serum medium and stimulated with $\text{TNF-}\alpha/\text{IFN-}\gamma$ for 24 hours. AMPK compound C inhibitor was added at $1\mu\text{g/ml}$ for 30 minutes prior to addition of adiponectin at $15\mu\text{g/ml}$ for 1 hour. Adiponectin treatment induced a decrease of transmigration, which was restored to normal controls levels with AMPK inhibition. AMPK inhibitor on its own did not change transmigration. Data are a pool of three experiments and were analysed using one-way ANOVA and Bonferroni's multiple comparisons post-test. ** $p\leq 0.01$, *** $p\leq 0.001$.

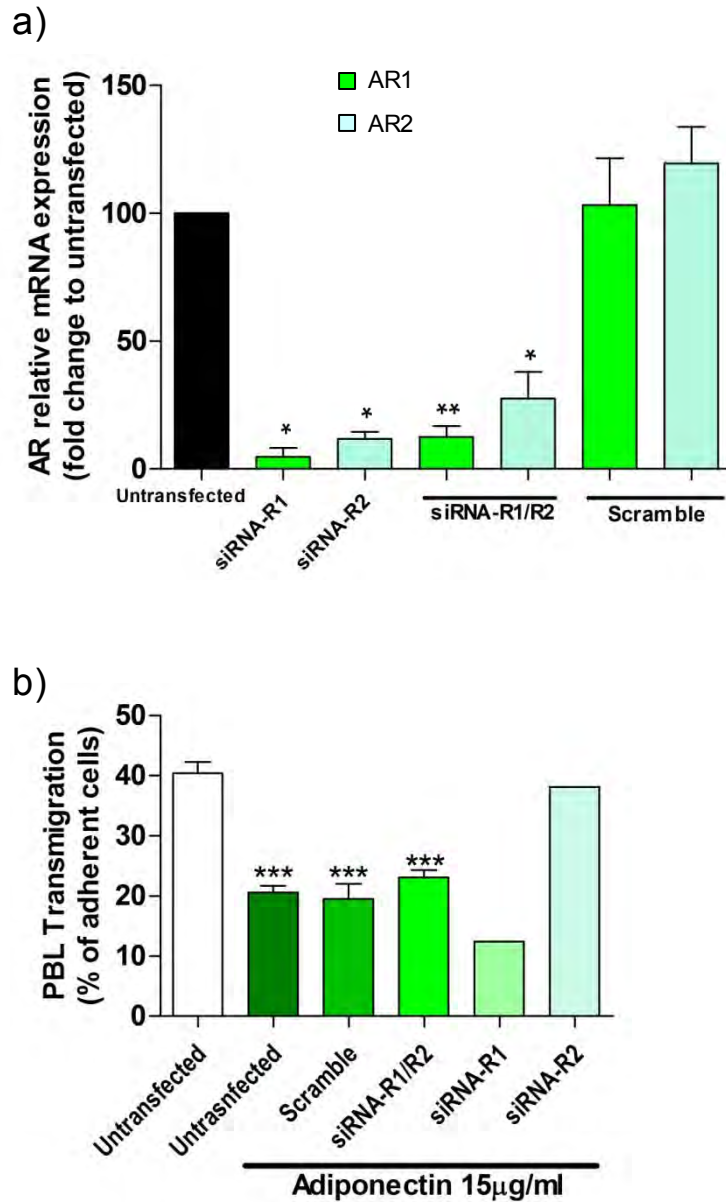


Figure 5-5: Effect of AR1 and AR2 knock-down on HUVEC

HUVEC were transfected with siRNA against AR1 (R1) and AR2 (R2) or scramble versions of the target siRNA. (a) Knock-down of both receptors was efficient in either single or double transfections, no decrease was observed with the control scrambled siRNA. (b) After 48 hours, HUVEC were stimulated with TNF- α /IFN- γ and adiponectin and PBL transmigration was measured by phase contrast videomicroscopy. The data are a pool of three experiments except for migration experiments on single receptor knock-down, and were analysed using one-way ANOVA and Bonferroni's multiple comparison post test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

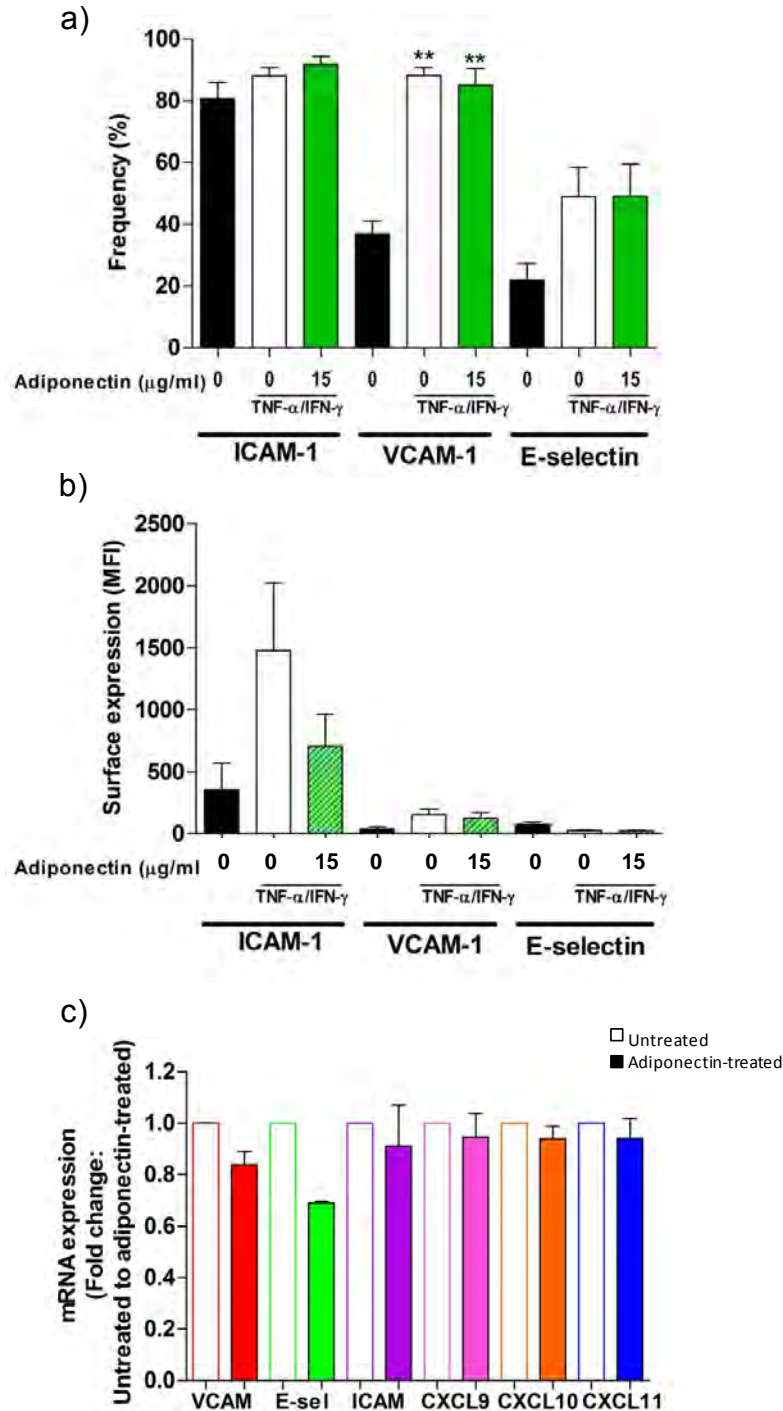


Figure 5-6: Adiponectin does not modulate expression of adhesion molecules and chemokines on HUVEC

HUVEC were cultured in absence or presence of adiponectin at 15μg/ml and adhesion molecules expression was measured by flow cytometry. (a) No significant changes were observed with adiponectin treatment for ICAM-1, VCAM-1 and E-Selectin frequency and (b) surface expression. Significant differences were found for VCAM-1 frequencies between unstimulated and stimulated HUVEC. (c) mRNA was extracted from TNFα/INFγ±adiponectin-treated HUVEC and adhesion molecules and chemokines expression was quantified by qPCR. Data are a pool of at least two experiments and were analysed using one-way ANOVA and Bonferroni's multiple comparisons post-test. **p≤0.01.

2.1.2. *Treatment of PBL with adiponectin inhibits lymphocyte transmigration*

The PBL subset used in our *ex-vivo* assays includes memory and naive CD4⁺ and CD8⁺ T cells, B cells, NK cells, CD56^{high} NK cells and NK T cells. Pang *et al.* have demonstrated that mainly B cells and NK cells express the adiponectin receptors in the lymphocytes pool. To further investigate the expression pattern of the receptor, we assessed AR1 and AR2 on memory (CD4⁺/CD8⁺CD45RO⁺) and naive (CD4⁺/CD8⁺CD45RO⁻) T cells, as well as B cells, NK cells (CD56⁺CD3⁻), CD56^{high}NK cells (CD56^{high}CD3⁻) and NK T cells (CD56⁺ CD3⁺) (**Figure 5-7**). Both receptors are expressed on B and NK cells (**Figure 5-8**). AR1 and 2 are also highly expressed on NK T cells and CD56^{high} NK cells. On all other T cells subsets, AR1 and AR2 expression is low (or absent) and there are no major difference between the memory and naive subsets.

Next, we aimed to investigate the effect of treating PBL with adiponectin for one hour prior to migration on TNF- α /IFN- γ -stimulated HUVECs in static conditions. Under these conditions, adiponectin considerably reduced transmigration, and this effect is dose-dependent (**Figure 5-9a, b**). Adiponectin had an EC50 of 0.94 μ g/ml in these experiments, calculated using non linear regression analysis (**Figure 5-9d**). The R² of the curve fitting to the data is 0.58, which indicates the goodness of the non linear regression fit is close to 1. The number of cells firmly adhered to the endothelium was reciprocally increased (**Figure 5-9c**) but no changes were observed on total adhesion or the velocity of lymphocyte migration underneath the endothelium (**Figure 5-10**). Similarly, adiponectin lowered PBL transmigration in flow conditions but no effect was observed on total levels of lymphocyte capture by the endothelium (**Figure 5-11**). Furthermore, there was a trend to elevated rolling for adiponectin-treated PBL.

We then checked whether the inhibition of PBL transmigration by adiponectin was mediated by AR1 and 2 signalling. PBL were pre-treated for 30 minutes with the AMPK

inhibitor compound C, followed by adiponectin for an hour. AMPK compound C reversed the effects of adiponectin and restored PBL transmigration to levels comparable with those observed on cytokine-treated HUVEC (**Figure 5-12**). These results support the concept that AR1/AR2 in lymphocytes is also important for transmigration of these cells. However, as mentioned earlier, AMPK is involved in numerous functions and signalling cascades (reviewed in Hardie, 2011). Therefore, we attempted to knock down the AR1/AR2 receptors on PBL using siRNA. Transfection of lymphocytes is challenging, so we started by optimising the transfection efficiency using a random siRNA coupled to FITC, which allowed detection of transfected cells by flow cytometry. Using this method, we obtained a higher transfection efficiency using the Amaxa technology compared to lipid-mediated transfection (Lipofectamine) (**Figure 5-13a**). Transfection of a pool of four siRNA targeting both receptors led to an average of 63% and 74% knock-down of AR1 and AR2 respectively (**Figure 5-13b**). Scramble siRNA were used as a negative control and also induced AR1 and AR2 knock-down (57% and 72% respectively). After comprehensive analysis of AR1 and AR2 expression profile on untransfected compared to transfected PBL, we realised that the B cell and NK cells subsets were missing in the transfected conditions (**Figure 5-14**). It appears that neither transfection methods is suitable for treating B cells and NK cells which we assume are killed by this process. Because B cells and NK cells express the highest levels of AR1 and AR2 in the PBL population, the knock-down at first analysis appeared to be efficient. However, in reality the loss of these receptors simply reflected the death of the receptor bearing cells. Because of the technical difficulties encountered, we could not assay the effect of AR1/ AR2 knock-down on adiponectin-mediated inhibition of PBL transmigration.

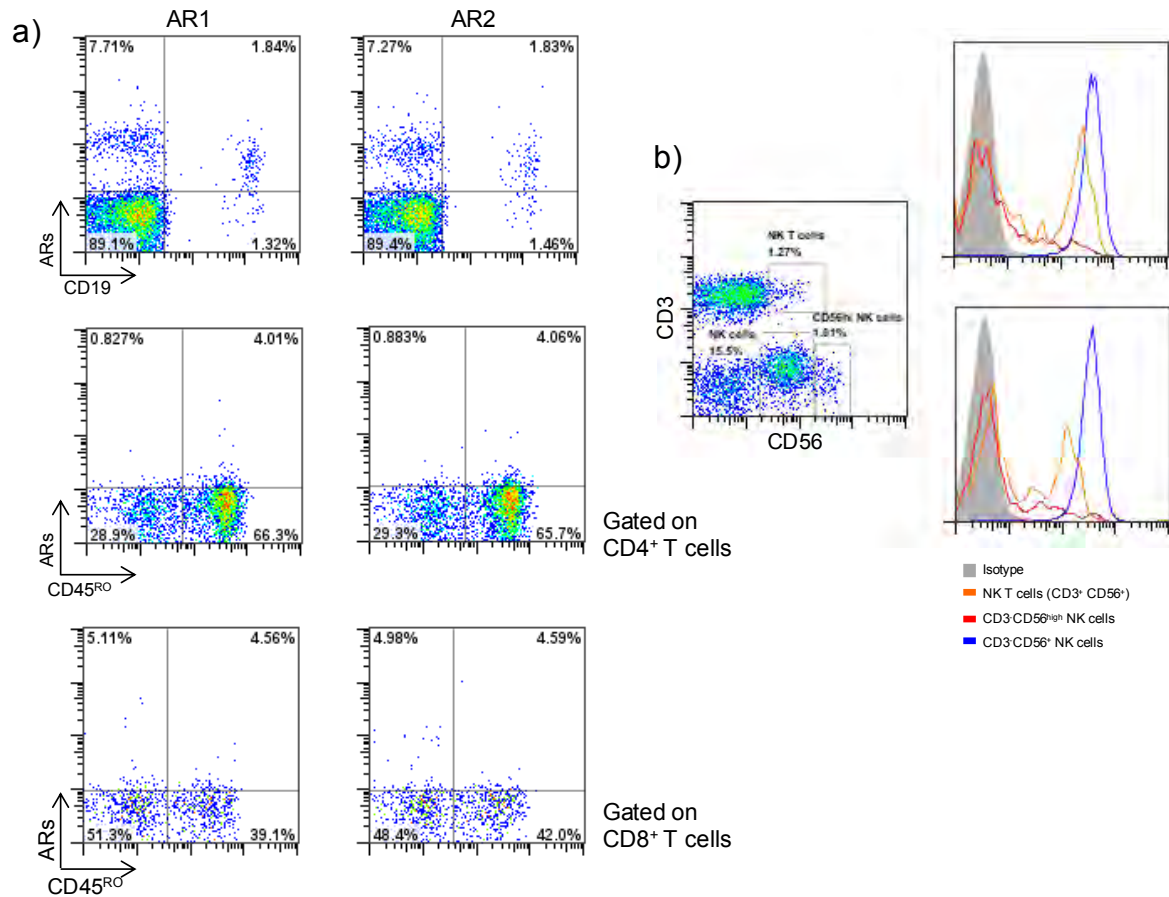


Figure 5-7: AR expression on PBL subsets

(a) Representative dots plots of AR1 and AR2 expression on PBL subsets. Viable cells were gated based on PI staining and PBL were gated on their forward/side scatter profile. B cells were identified with CD19 labelling, CD4⁺ and CD8⁺ T cells were gated and CD45^{RO} allowed identifying the memory and naive subsets. (b) The different NKs populations were identified using CD3 and CD56. AR expression was determined based on the isotype control for each subset. Numbers represent the quadrants and gates frequencies. The data are representative of seven healthy controls.

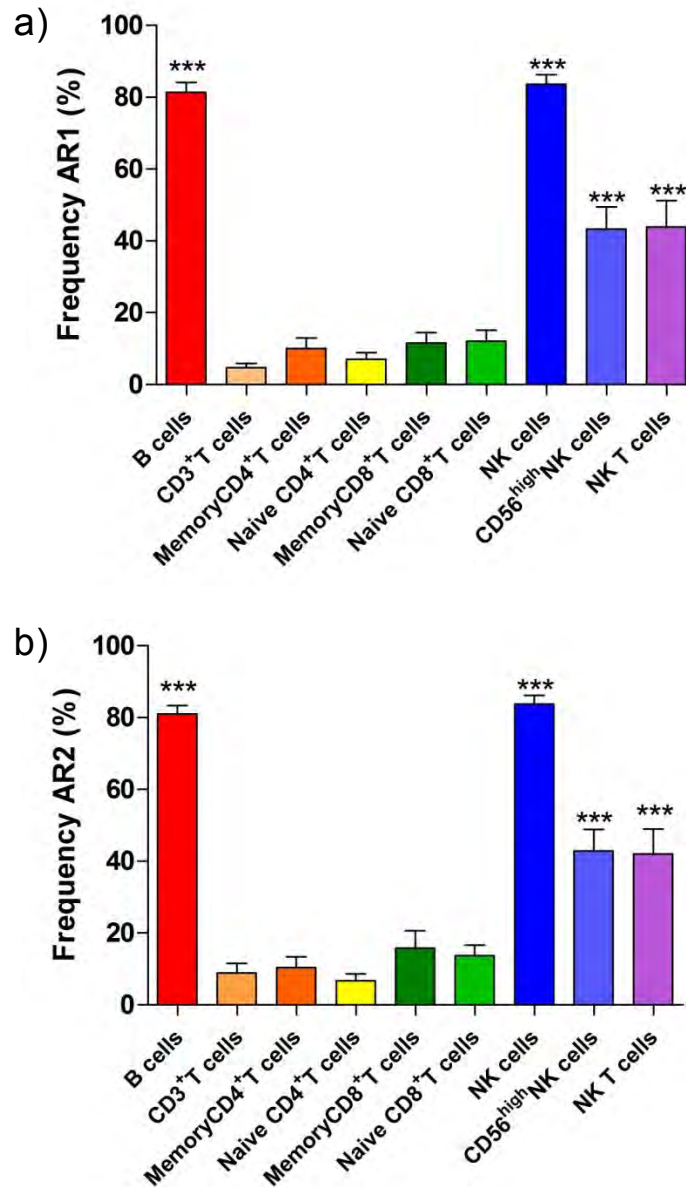


Figure 5-8: ARs frequencies on PBL subsets

Viable cells were gated based on PI staining and PBL were gated on their forward/side scatter profile. Each subset was gated based on their markers expression: CD19, CD56, CD3, CD4, CD8 and CD45^{RO}. AR1 (a) and AR2 (b) expression was calculated by subtraction against the negative isotype control. The data are mean±SEM and are a pool of seven healthy controls. Data were analysed using one-way ANOVA and Bonferroni's multiple comparisons post-test. ***p≤0.001.

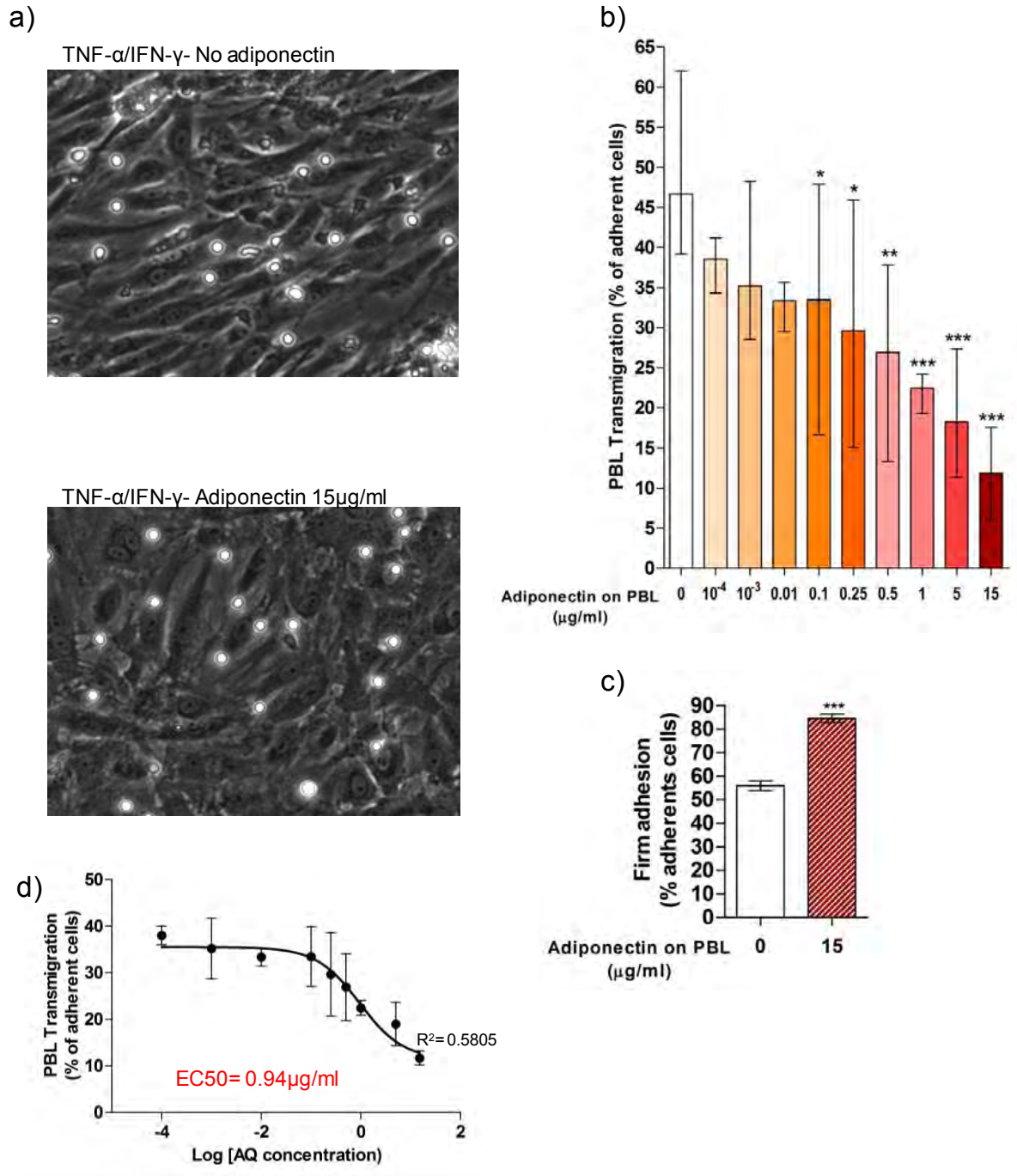


Figure 5-9: PBL treated with adiponectin have reduced transmigration capacities in static conditions

HUVEC were cultured in low serum medium and stimulated with TNF- α /IFN- γ for 24 hours in absence of adiponectin. PBL were isolated and treated with adiponectin at 0.0001 to 15 μ g/ml during one hour. (a) PBL transmigration was significantly reduced from 43% (top) to 8.33% (bottom) with adiponectin treatment at 15 μ g/ml. (b) This effect is dose dependent and (c) firm adhesion of adiponectin-treated PBL is increased. Data are a pool of at least three experiments and were analysed using t-test and one-way ANOVA and Dunnett's multiple comparisons post-test. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. An EC₅₀ of 0.94 μ g/ml was calculated using non linear regression analysis.

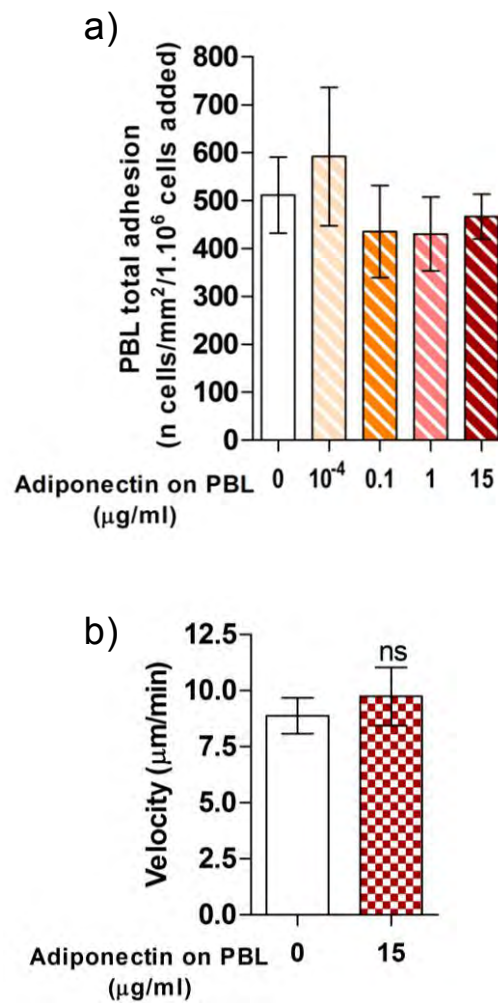


Figure 5-10: Adiponectin on PBL does not influence total recruitment and velocity

HUVEC were cultured in low serum medium and stimulated with TNF- α /IFN- γ for 24 hours in absence adiponectin. There were no significant changes in (a) total adhesion and (b) velocity of the transmigrated cells after adiponectin treatment of PBL. Data are a pool of at least three experiments and were analysed using t-test and one-way ANOVA and Dunnett's multiple comparisons post-test.

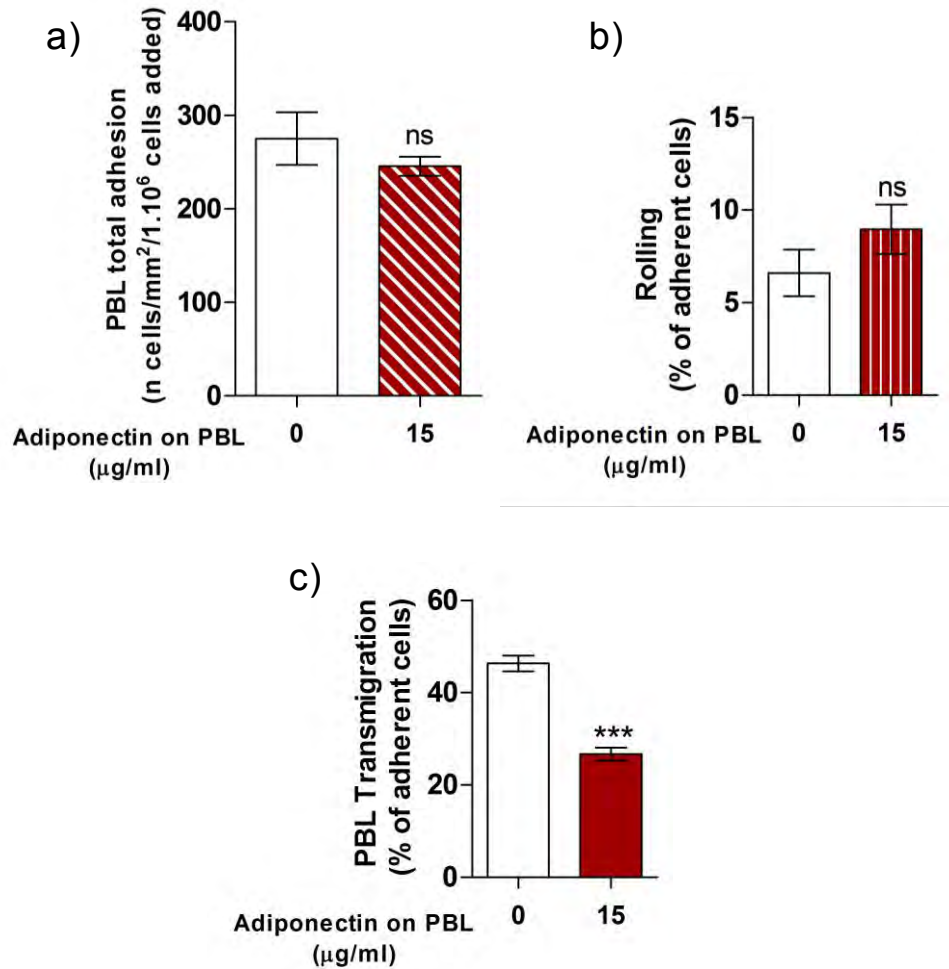


Figure 5-11: PBL treated with adiponectin have reduced transmigration capacities in flow conditions

HUVEC were cultured in low serum medium in Ibidi slides and stimulated with TNF- α /IFN- γ for 24 hours in absence of adiponectin and PBL were pre-treated with adiponectin at 15 μ g/ml during one hour prior to migration. (a) There is no change in total adhesion and (b) a trend to increased rolling. (c) PBL transmigration was reduced from an average of 46% to 26% with adiponectin treatment. Data are a pool of three experiments realised in duplicates and were analysed using t-test. *** $p \leq 0.001$.

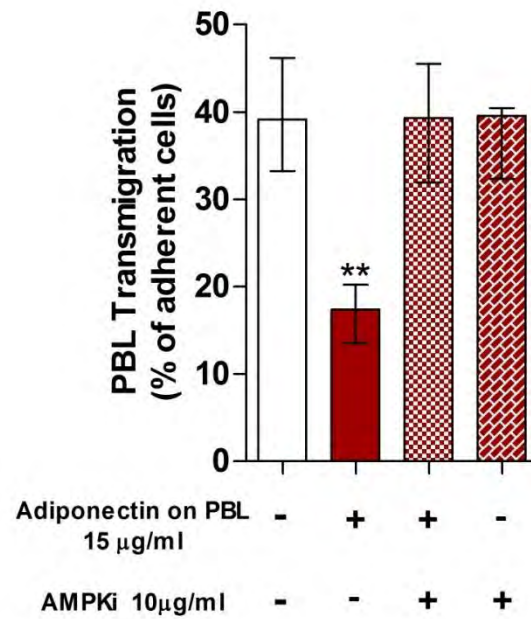


Figure 5-12: AMPK inhibition restores PBL transmigration

HUVEC were cultured in low serum medium and stimulated with $\text{TNF-}\alpha/\text{IFN-}\gamma$ for 24 hours. AMPK compound C inhibitor was added at $10\mu\text{g/ml}$ for 30 minutes prior to addition of adiponectin at $15\mu\text{g/ml}$ for one hour. Adiponectin treatment induced a decrease of transmigration, which was restored to normal controls levels with AMPK inhibition. AMPK inhibitor on its own did not change transmigration. Data are a pool of three experiments and were analysed using one-way ANOVA and Dunnet's multiple comparisons post-test. ** $p\leq 0.01$, *** $p\leq 0.001$.

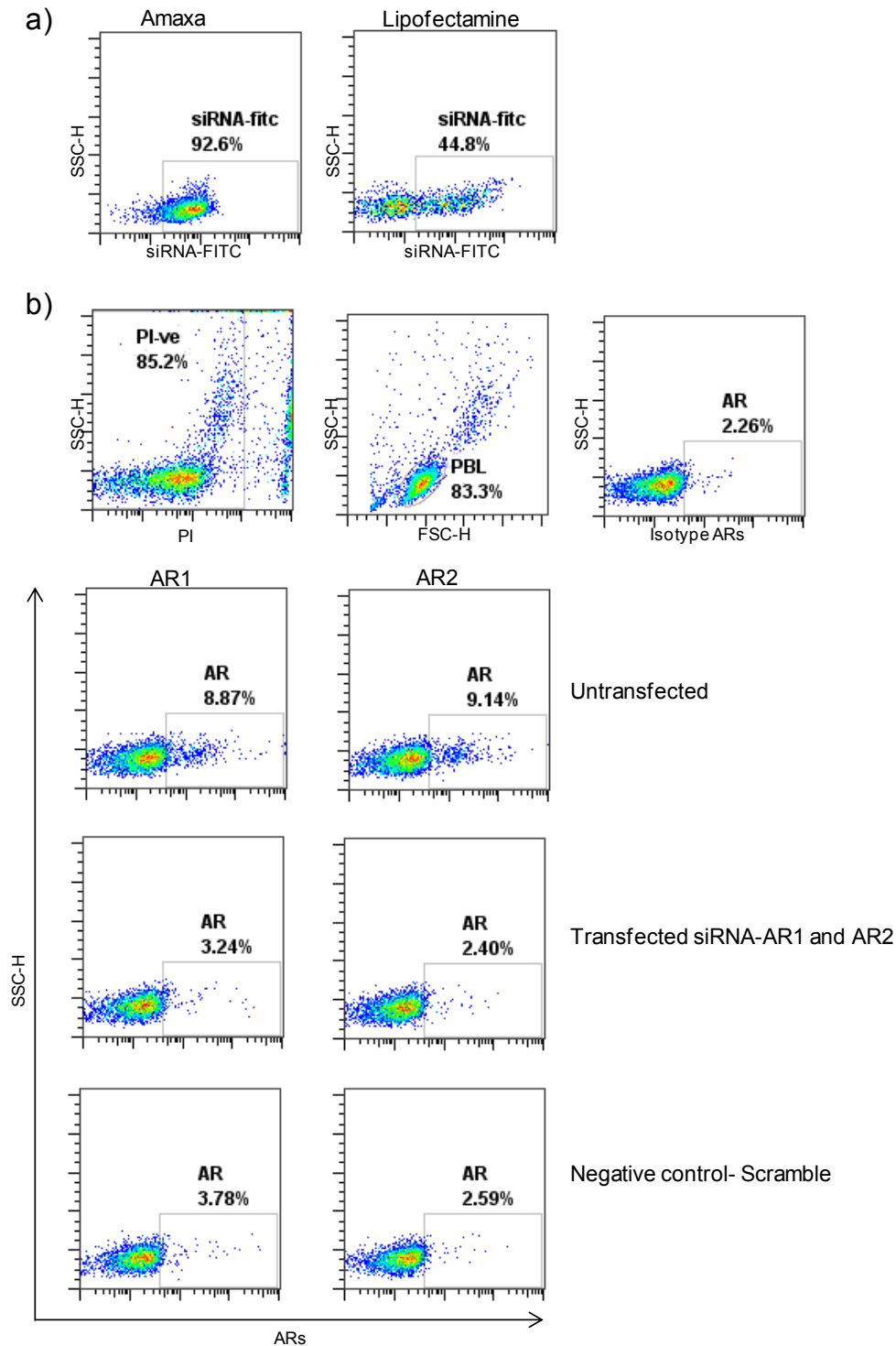


Figure 5-13: Transfection of PBL with siRNA targeting AR1 and AR2

(a) PBL were transfected with siRNA-FITC using Amaxa or Lipofectamine techniques. Amaxa transfection leads to higher siRNA-FITC entry into the cells. (b) PI staining shows good viability of transfected cells and PBL were gated on forward/side scatter to determine ARs expression by setting the gate on the isotype control. (c) PBL were then transfected with a pool of four siRNA targeting AR1 or AR2 and both receptor expression was measured by flow cytometry. AR1 and AR2 expression are reduced on cells transfected with siRNA against AR1 or AR2, but the scramble negative control also induced ARs knock-down. The data are representative of at least three experiments.

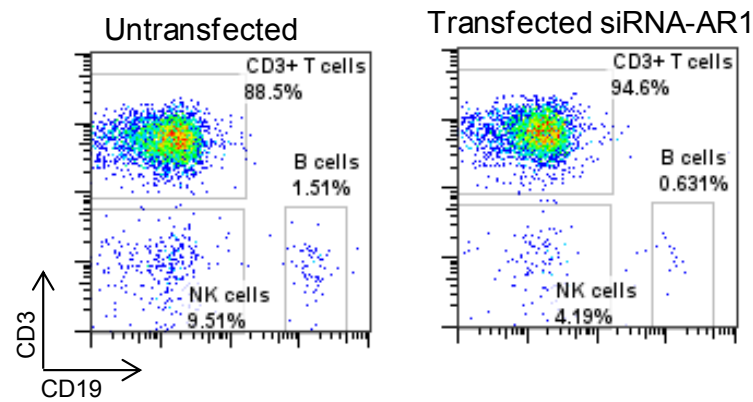


Figure 5-14: AR knock-down reflects B and NK cell death

Numbers represent gate frequencies. PBL were transfected with siRNA against AR1 using the Amaxa technology. The frequencies of CD19⁺ B cells and NK cells (CD3⁻CD19⁻) were reduced when cells were transfected with siRNA (right dot plot) compared to untransfected cells (left dot plot).

2.2. Adiponectin-mediated inhibition of transmigration is altered in T1D

Finally, we examined whether adiponectin could modulate transmigration of PBL from patients with T1D. Clinical parameters of healthy controls (HC) and T1D subjects are described in **Table 5-1**. Thirteen Caucasian or Asian age and BMI matched female and male subjects were recruited from the Diabetes Clinics of the Birmingham University Hospital along with twelve healthy laboratory colleagues. The cohort exhibited no significant differences for age and BMI between the HC and T1D groups. Most patients with T1D had established diabetes but two were newly diagnosed.

	HC	T1D
N	12	13
n Female/Male	6/6	5/8
Age (years)	42.07 ± 14.98	46.77 ± 11.3
BMI (kg.m ²)	23.56 ± 8.3	25.87 ± 4.53
Duration of diabetes (years)	NA	18.8

Table 5-1: Clinical parameters of the patients included in the study

Data are represented as Mean ± SD and analysed using non-parametric t-test. No significant differences for age and BMI were found between HC and T1D subjects.

First, we analysed the expression profile of AR1 and AR2 using flow cytometry on each PBL subsets in both groups. PBL were gated on PI, forward/side scatter and pulse width plots. The expression of both receptors was reduced on PBL from patients with T1D compared to “age matched” healthy controls (**Figure 5-15**). The different PBL sub-populations were identified by CD19 for B cells, CD3 for T cells, CD3⁺CD56⁺ for NK T cells, CD3⁻CD56⁺ for NK cells and CD3⁻CD56^{high} for CD56^{high} NK cells. Closer analysis of the expression of both receptors on B cells and T cells revealed no difference in AR1 and AR2 frequency between HC and T1D (**Figure 5-16**). In both groups however, the expression of AR1 and AR2 was very high on B cells compared to T cells, which confirms earlier

findings (Pang, Narendran, 2008). Interestingly, NK T cells exhibited a significantly lower expression of both receptors in T1D (**Figure 5-17**).

We went on to explore whether adiponectin modulates transmigration of PBL from patients with T1D at the same magnitude to healthy controls. The behaviour of PBL in response to adiponectin was consistent with previous results in the HC group (**Figure 5-18**). However, the adiponectin inhibition of transmigration was impaired with PBL from T1D patients (**Figure 5-19**).

Finally, as we observed a reduction in adiponectin receptors in T1D, we speculated whether this could be associated with the reduced effect of adiponectin observed on PBL transmigration. Indeed, the levels of both AR1 and AR2 are positively correlated with the percentage of inhibition of transmigration by adiponectin, to the point where the T1D and HC cohorts could be separated by plotting these parameters on separate axis (**Figure 5-20**).

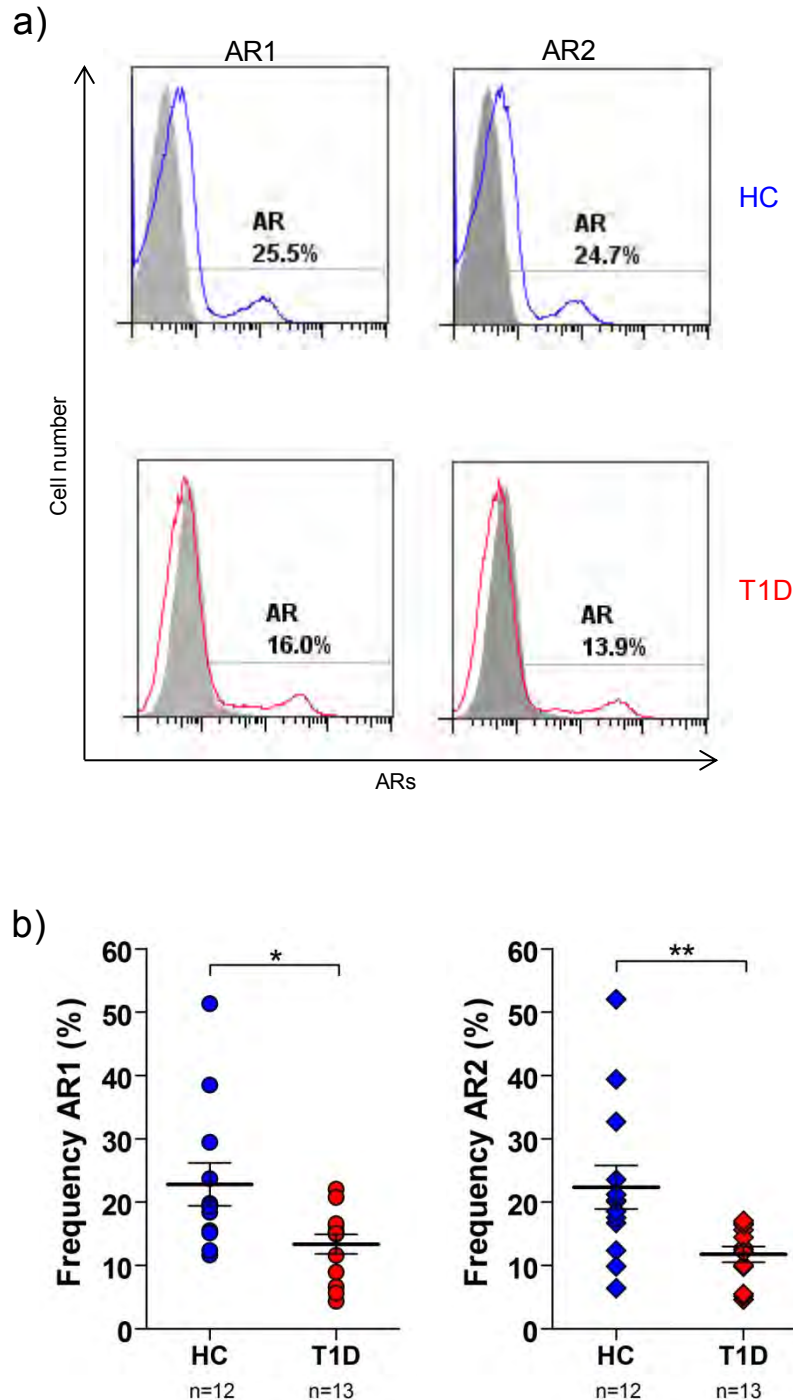


Figure 5-15: PBL from patients with T1D express less adiponectin receptors

(a) Representative histograms of AR1 and AR2 expression on PBL from healthy controls (top panel) and patients with T1D (bottom panel). (b) AR1 and (c) AR2 frequencies were determined for each subject by setting the gate on the isotype control and subtracting the isotype frequency from the AR staining. For example, the isotype frequency for HC (a, top panel) is 2.02% and AR1 is 25.5%. So the calculated AR1 expression is: $25.5\% - 2.02\% = 23.48\%$. Data are represented as mean \pm SEM and were analysed using t-test or Mann Whitney t-test when data did not pass the Kolmogorov-Smirnov normality test. * $p \leq 0.05$, ** $p \leq 0.01$.

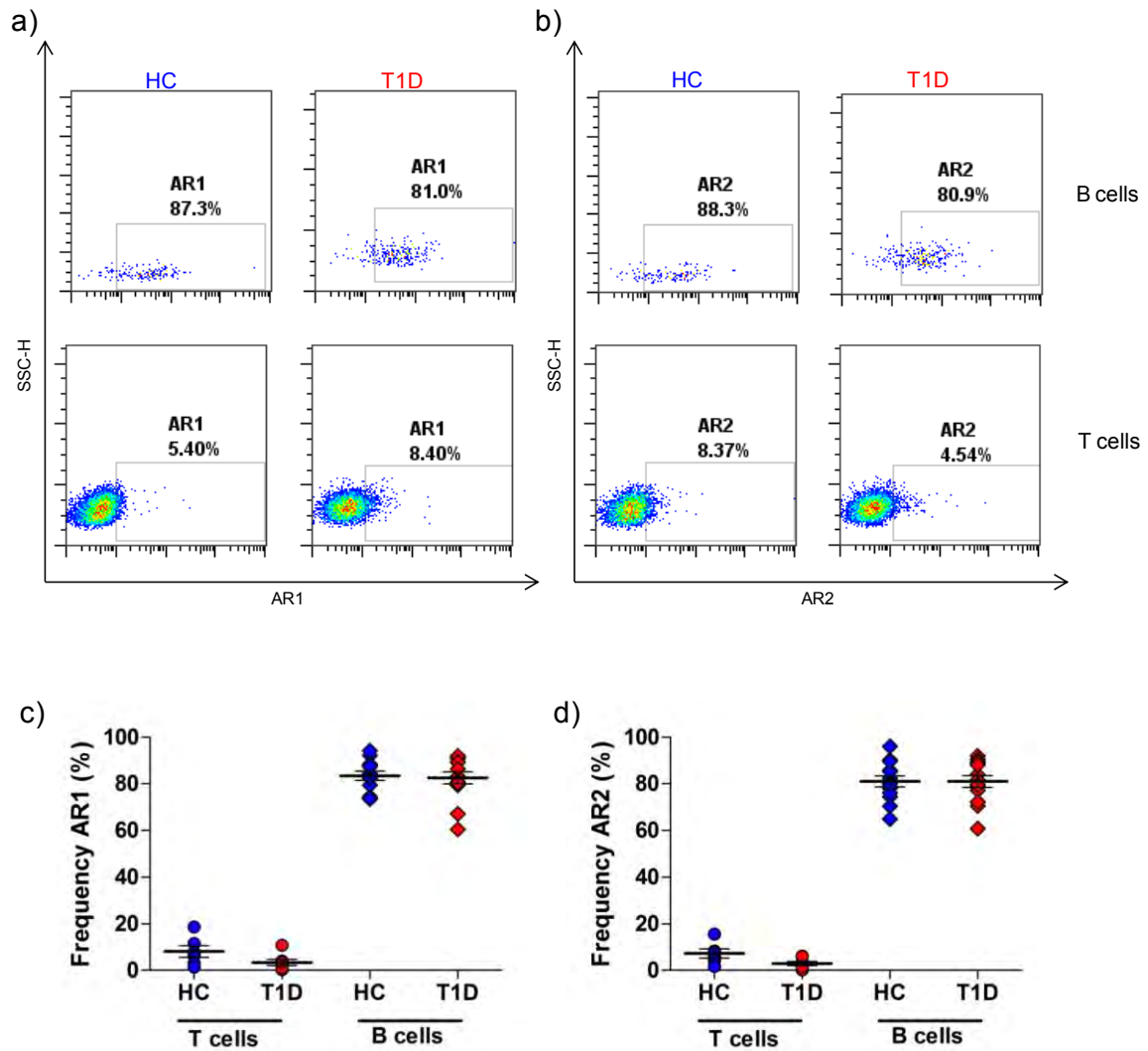


Figure 5-16: AR expression is not affected on B and T cells from patients with T1D

Representative dot plots of (a) AR1 and (b) AR2 expression on B and T cells from healthy controls and patients with T1D. B cells were identified by their CD19 expression and T cells by CD3. (c) AR1 and (d) AR2 frequencies were determined on both populations for each subject by setting the gate on the isotype control and subtracting the isotype frequency from the AR staining. No significant differences were found between the patients with T1D and the healthy controls. Data are represented as mean \pm SEM and were analysed using t-test. Data are a pool of seven subjects for both groups.

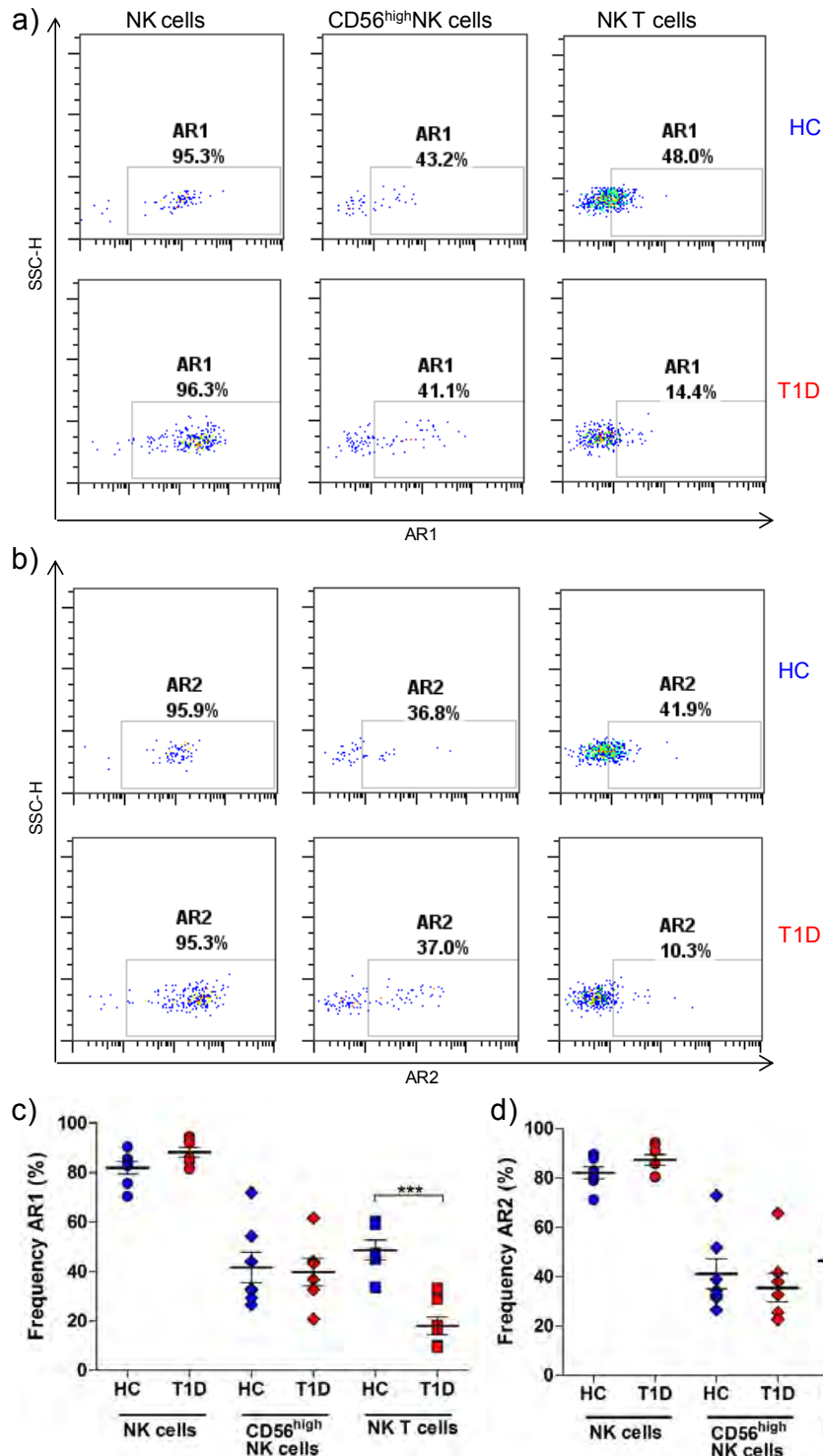


Figure 5-17: AR1 and AR2 expression is reduced on CD56^{high} NK cells

Representative dot plots of (a) AR1 and (b) AR2 expression on NK cell subsets from healthy controls and patients with T1D. NK cells, CD56^{high} NK cells and NK T cells were identified by their expression of CD3 and CD56 as described earlier. (c) AR1 and (d) AR2 frequencies were determined on the three populations for each subject by setting the gate on the isotype control and subtracting the isotype frequency from the AR staining. Data are represented as mean±SEM and were analysed using t-test. Data are a pool of seven subjects for both groups. ***p≤0.001.

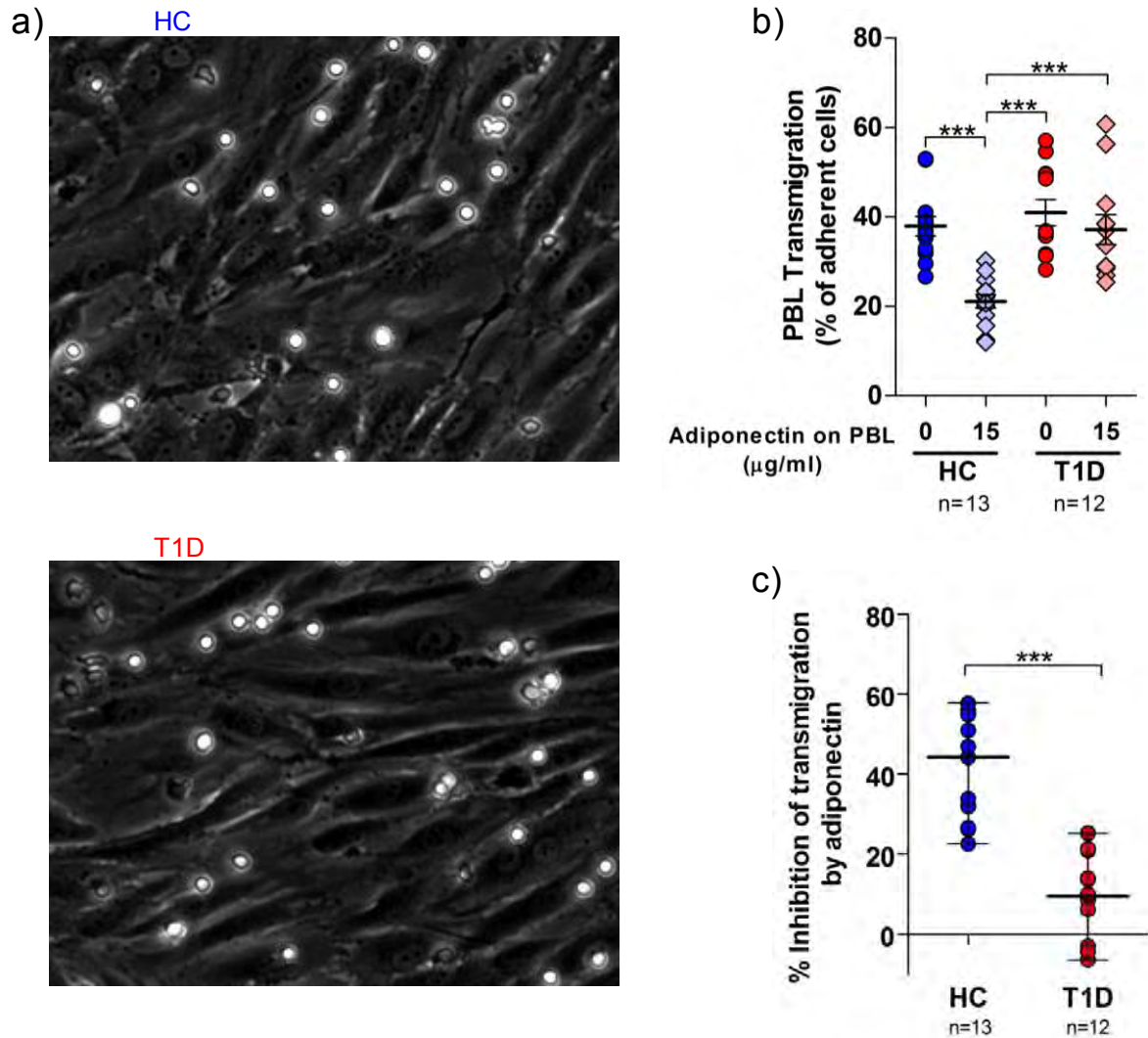


Figure 5-18: PBL from T1D patients are released from the inhibitory effect of adiponectin on lymphocyte transmigration

HUVEC were cultured in low serum medium and stimulated with $\text{TNF-}\alpha/\text{IFN-}\gamma$ for 24 hours in absence of adiponectin. PBL were isolated and treated with adiponectin at $15\mu\text{g/ml}$ for one hour. (a) PBL transmigration was significantly higher in patients with T1D: 31% of cells transmigrate (bottom) compared to 18% in HC (top) with adiponectin treatment at $15\mu\text{g/ml}$. (b) This indicates that adiponectin-mediated inhibition of PBL transmigration is reduced in T1D. (c) The percentage of inhibition was calculated by dividing the percentage of transmigration with adiponectin treatment by the percentage of transmigration of untreated PBL. $n=13$ for HC group and $n=12$ for T1D group. Data were analysed using t-test and one-way ANOVA and Bonferroni's multiple comparisons post-test. *** $p\leq 0.001$.

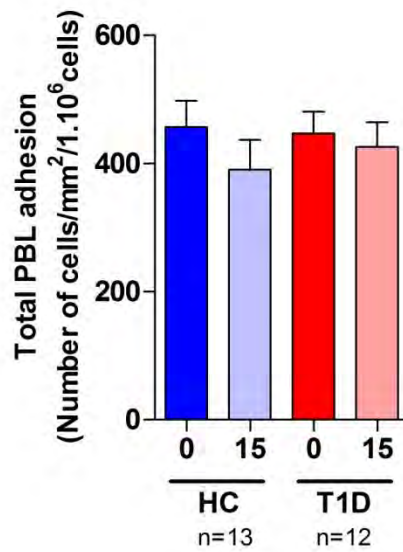


Figure 5-19: Total PBL adhesion is unchanged in T1D

HUVEC were cultured in low serum medium and stimulated with TNF- α /IFN- γ for 24 hours in absence adiponectin. There were no significant changes in total adhesion between HC and T1D with or without adiponectin. Data are a pool of at least three experiments and were analysed using non-parametric one-way ANOVA and Bonferroni's multiple comparisons post-test.

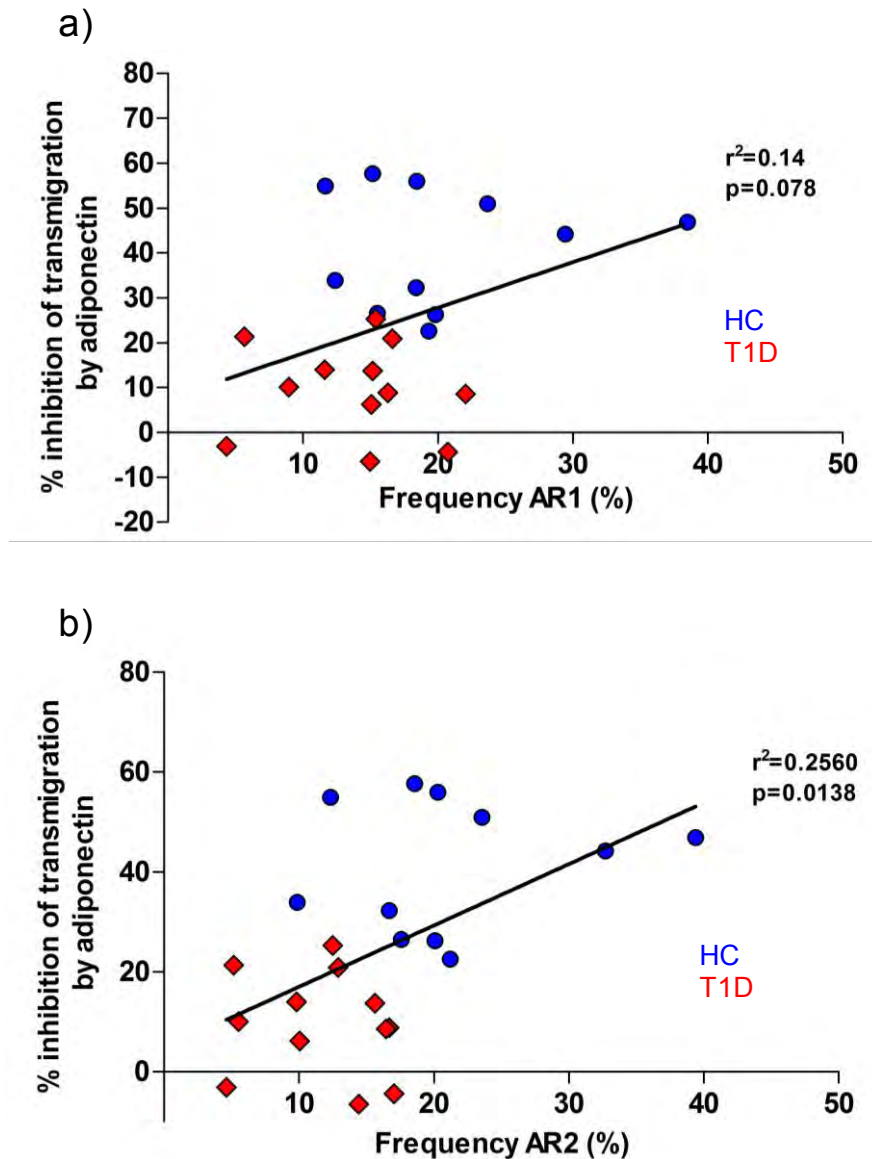


Figure 5-20: Levels of adiponectin receptors correlate with the capacity of adiponectin to inhibit PBL transmigration

Correlations between frequency of (a) AR1 and (b) AR2 with the percentage of adiponectin inhibition of PBL transmigration were determined using linear regression analysis.

3. Discussion

We studied the effect of adiponectin on transmigration in order to examine the functional relevance of the different expression levels of adipokine receptors in T1D. Using an established model of lymphocyte migration, we show that adiponectin can strongly inhibit PBL transmigration by direct action on HUVEC and also on PBL. Additionally, we demonstrated that adiponectin-mediated inhibition of PBL transmigration is altered in T1D and this was significantly correlated with the level of adiponectin receptors expressed on PBL.

- **Measurement of PBL transmigration *in vitro***

Migration of PBL through endothelial cells has previously been studied using HUVEC, in both static and flow-based assays. HUVEC are well characterised, relatively easy to acquire and express the relevant adhesion molecules and chemokines required for efficient PBL recruitment. In static assays, PBL are allowed to settle on HUVEC monolayers in the absence of physical forces (Oppenheimer *et al.*, 1990; McGettrick *et al.*, 2007 and 2009). Static assays are relatively easy and quick to set up on clear plastic and require stimulation of HUVEC with both TNF- α and IFN- γ to support efficient recruitment of PBL (McGettrick *et al.*, 2009). Under these conditions, adhesion and transmigration are observable as early as 6-8 minutes after addition, using phase-contrast videomicroscopy. This is in comparison with widely used transwell systems in which PBL only migrate efficiently across endothelium and transwell membrane after about 18 hours (McGettrick *et al.*, 2009). Indeed, the transwell system is not very physiological as it is unlikely that PBL takes so long to cross the endothelium *in vivo*. The only disadvantage of static assays on clear plastic is that it is not possible to analyse dynamic capture process as capture from flow and rolling cannot be distinguished. That is why we also confirmed our findings in flow assays. In this system, PBL

are perfused on the HUVEC monolayer at a specific shear stress (0.1Pa) (Luscinskas *et al.*, 1995; Bahra *et al.*, 1998; McGettrick *et al.*, 2009). This allows the quantification of total capture, rolling, firm adhesion and transmigration in a more physiological context.

Many studies have used T cell lines or stimulated T cells in migration assays. In our study, we studied migration of unstimulated PBL because evidences suggest that PBL can directly be recruited from the circulation during inflammation *in vivo* (Cose *et al.*, 2007). In addition, TNF- α and IFN- γ stimulation provides the appropriate signals supporting PBL migration as it would in an inflammatory context. Indeed, HUVEC stimulation induces up-regulation of ICAM-1, VCAM-1 and E-selectin expression as well as presentation of chemokines such as CXCL9, 10 and 11, required for efficient lymphocyte migration (Piali *et al.*, 1998; Mazanet *et al.*, 2000, McGettrick *et al.*, 2009).

- **Effect of adiponectin on PBL transmigration**

Previous studies in the adiponectin knock-out mouse model have demonstrated a role for adiponectin in regulating leukocyte migration. In this section, stimulated endothelium was treated with adiponectin for 24 hours or PBL were treated with adiponectin for one hour, before the adhesion assay. In both conditions, transmigration was drastically reduced, but the magnitude of the inhibition was more pronounced when the PBL were pre-treated with adiponectin.

Previous studies have linked the protective effect of adiponectin on vascular health to its capacity to preserve endothelial cell function. Indeed, low levels of adiponectin correlate with cardiovascular disease (Goldstein *et al.*, 2007; Zhu *et al.*, 2008) by causing impairment of endothelial-dependent vasodilatation *in vivo* (Ouchi *et al.*, 2003; Shimabukur *et al.*, 2003; Tan *et al.*, 2004; Maruyoshi *et al.*, 2005). These effects have been associated with regulation of endothelial cell derived nitric oxide production by adiponectin. Indeed, aortic rings from

the adiponectin knock-out mouse show lower capacity to produce NO (Cao *et al.*, 2009). Such effects have also been reported in bovine endothelial cells treated with adiponectin (Chen *et al.*, 2003). Particularly relevant to our study, Ouedraogo *et al.*, have reported elevated rolling and adhesion in the adiponectin knock-out mouse model. This was shown both *in vivo*, using intravital microscopy and *ex-vivo* using isolated mouse aorta. In the aortic preparations, monocytes adhesion was significantly increased in the knock-out tissue compared to wild-type controls, and addition of adiponectin restored normal levels of rolling and adhesion in this assay and in the intravital model. In addition, this study linked the adiponectin-mediated regulation of leukocyte recruitment to the control of NO levels. The authors implied an effect of this pathway on E-selectin and VCAM-1 expression by the endothelium. This was also shown using human endothelial cells when adhesion of THP-1 monocytic cell line was the functional readout (Ouchi *et al.*, 2003). To our knowledge the role of adiponectin in the regulation of lymphocyte trafficking has not been investigated with the exception of one study where lymphocytes were co-incubated with adiponectin and motility was observed in collagen gels (Lang *et al.*, 2009). In these conditions, motility was not changed for CD8⁺ T cells.

In our system, we show for the first time that adiponectin inhibits lymphocyte transmigration across HUVEC. In this case, we pre-treated the HUVEC for 24 hours, similarly to previous studies (Ouchi *et al.*, 2003). In this static assay, we observed a concomitant increase of firmly adhered PBL but no changes on total adhesion or migration velocities underneath the endothelium. These observations were reproduced in a flow based assay, which also showed modest changes in the levels of rolling cells. In addition, we found that inhibition of AMPK, a crucial intermediate of the adiponectin signalling cascade, inhibits the effects of adiponectin on lymphocyte transmigration. Although blockade of AMPK has widely been used to show a link between adiponectin and its receptors signalling (Son *et al.*, 2008), this is not an optimal test, as AMPK is involved in many other cellular functions, e.g

metabolism, regulation of mitochondrial biogenesis and disposal, cell polarity, cell growth and cell differentiation (Hardie *et al.*, 2011). As no receptor antagonist is available, we aimed to knock-down the adiponectin receptors on HUVEC using siRNA as we have shown high expression on these cells. Knock-down of the receptors was efficient and we showed that the effect on HUVEC seem to be mediated by AR2. However, this experiment was only performed once due to the scarcity of HUVEC at this time.

Conversely to previous studies, we did not discern a modulation of E-selectin, ICAM-1, VCAM-1, or the IFN- γ inducible chemokines, CXCL9, 10 and 11 on the endothelium in response to adiponectin, suggesting that another mechanism exists for regulating lymphocyte trafficking. We have not yet understood the mechanism of action of adiponectin on HUVEC, but we believe that adiponectin is altering either the firm adhesion of lymphocytes on the endothelium or the lipid signalling leading to trans-endothelial migration. The levels of adhesion per se, and the fraction of cells rolling are not altered, suggesting that the function of E-selectin is unaltered. Firm adhesion is only possible if the right chemokines signals activate LFA-1 ($\alpha_L\beta_2$) and VLA-4 ($\alpha_4\beta_1$) integrins (Springer, 1994) so that they bind their endothelial cell borne receptors VCAM-1 and ICAM-1. However, in the absence of any discernable changes in chemokine expression we anticipate that adiponectin might be acting on the signalling process that modulates integrin function in lymphocytes.

Recruitment of lymphocytes is critical for the pathophysiology of vascular diseases (Joussen *et al.*, 2004; Krieglstein *et al.*, 2001). However, in physiological conditions, the recruitment of lymphocytes is minimal. Upon inflammation, adhesion molecules, selectins and chemokines are up-regulated at the surface of the endothelium with a concomitant increase of integrins expression on the circulating lymphocytes (Carlos *et al.*, 1994). In conditions such as obesity, the perturbation of the physiology of adipose tissue causes up-

regulation of pro-inflammatory molecules and a decrease in secretion of anti-inflammatory mediators such as adiponectin (Festa *et al.*, 2001). Consequently, prolonged exposure of the endothelium to these pro-inflammatory markers induces perturbations of endothelium homeostasis. Obesity is clearly associated with lower levels of adiponectin compared to healthy controls, which might contribute to the loss of control of leukocytes migration (Arita *et al.*, 1999; Hotta *et al.*, 2000; Pang *et al.*, submitted). Inflammation is consequently maintained and can cause long-term complications. In our study, we were interested in T1D. Interestingly, this condition is not associated with changes in circulating adiponectin levels compared to healthy controls (Pang *et al.*, submitted). However, T1D is also a chronic inflammatory disease. Here, we did not assess whether adiponectin could modulate the transmigration of patient PBL across endothelial cells treated with adiponectin. However, this would be worth investigating in the future. In addition, it would be interesting to test adiponectin on islet endothelium as these differ from our macrovascular model (Vajkoczy *et al.*, 1995; Olsson *et al.*, 2006).

In T1D, we found that expression of the adiponectin receptors on lymphocytes is lower compared to healthy controls. We have shown that lower levels of both receptors are negatively correlated to the capacity of adiponectin to inhibit lymphocyte transmigration. In other terms, low levels of adiponectin receptors reduce the sensitivity of lymphocytes to adiponectin. Although this is novel in the field of leukocytes recruitment to the endothelium, this was observed in our group before, when the co-stimulatory capacities of DCs were assayed (Pang *et al.*, submitted). In this section, we also show for the first time that adiponectin can strongly inhibit lymphocyte trans-endothelial migration by action on the PBL themselves. These findings were also reproduced in flow conditions and inhibition of AMPK signalling rescued PBL transmigration from the adiponectin inhibition. We also transfected PBL with siRNA duplexes against the adiponectin receptors. However, expression of PBL

sub-populations revealed that transfection caused a dramatic reduction in the number B lymphocytes and NK cells, which were probably killed by this process. We accept this as a limitation in this study, as we have not definitively confirmed that the effect of adiponectin on PBL is mediated by AR1 and/or AR2. This would be worth confirming in the future especially since studies have revealed that other receptors to adiponectin, such as T-cadherin, exist (Hug *et al.*, 2004). Recent evidence shows that T-cadherin signals through AMPK to mediate the cardioprotective effects of adiponectin (Denzel *et al.*, 2010). Indeed, levels of adiponectin dramatically increase in absence of T-cadherin, and studies have reported mutations on the T-cadherin gene that affects adiponectin levels are associated with risk of metabolic syndrome, T2D and stroke in the Chinese, Korean and Philipino populations (Chung *et al.*, 2011; Wu *et al.*, 2010; Jee *et al.*, 2010). This suggests that in our system, T-cadherin could also mediate the adiponectin-mediated inhibition of lymphocyte transmigration. However, we have failed to detect T-cadherin gene expression on whole PBMC or sorted T cells (data not shown). However, HUVEC exhibited high levels of T-cadherin (dissertation Aled Benbow- Intercalating BMedSc student). We believe that T-cadherin might be relevant for the effects of adiponectin observed by treating endothelium with this adipokine. As a matter of fact, some believe that T-cadherin and adiponectin receptors act in synergy to mediate vascular protection. This view is based on the evidences that absence of both AR1 and T-cadherin independently cause vascular problems because of the lack of adiponectin signalling (Denzel *et al.*, 2010; Iwabu *et al.*, 2010).

Although T cadherin may play a role on endothelial cells, our study shows that the capacity of adiponectin to inhibit PBL transmigration positively correlates with adiponectin receptor levels on PBL. This corroborates the involvement of these receptors in mediating adiponectin effects in our system. However, we acknowledge some limitations in the study of AR1/2 levels in T1D compared to HC. First, although we managed to match healthy controls

to the patients with T1D for BMI and age, the subjects were not matched for gender and ethnicity. Gender and ethnicity induce variations in adiponectin levels and may affect the receptor levels (Barnes *et al.*, 2008; Mente *et al.*, 2010). Another limitation of this study is the absence of adiponectin receptors surface expression measured by MFI. At the time of sample processing, we faced equipment problems and we had to use a different flow cytometer to acquire the data. This consequently changed the MFI as this varies between machines depending on the lasers intensities and the parameters used. Therefore, we cannot compare surface expressions between the different subjects. We could also expect that adiponectin receptors expression would be lower on B cells from patients with T1D, therefore lowering the response of B cells to adiponectin. But this was not observed in our small cohort of patients. However, we found a significant decrease of adiponectin receptors expression on B cells in T1D compared to healthy controls in the subsequent cohort of patients that we used for the LEPR study (Pang PhD, data not shown). Indeed this cohort recruited more patients and controls, which were matched for gender, age and ethnicity. This level of control lacked in our cohort and this might have introduced bias in the receptor levels (Barnes *et al.*, 2008; Mente *et al.*, 2010).

Finally, it is worth indicating that in this study we used a human recombinant adiponectin kindly donated by Soren Tullin (NovoNordisk). This source of high molecular weight adiponectin has exceptionally low contents of endotoxins (0.6-3.21EU/mg) compared to commercially available sources (≥ 40 EU/mg) (Turner *et al.*, 2009). Indeed, it is crucial to use such a clean source of adiponectin as LPS contents of the commercially available sources have been associated with some contradictory data (Turner *et al.*, 2009).

- **Conclusions**

We demonstrate that adiponectin prevents lymphocyte trans-endothelial migration in both HUVEC and PBL treatment conditions. This effect was dose-dependent in both circumstances and was reversed by AMPK inhibition. The adiponectin effect on PBL was lost in patients with T1D where adiponectin receptors expression is lower. This is consistent with our hypothesis that levels of adiponectin receptors determine the sensitivity of immune cells to adiponectin. The mechanism of adiponectin effect remains unclear, and will be explored in the next chapter.

6.CHAPTER 6- THE ADIPONECTIN- DEPENDENT INHIBITION OF T CELL MIGRATION IS MEDIATED BY B CELLS

1. Introduction

In agreement with previous data from our group, we found that AR1 and AR2 are mainly expressed on B cells, NK cells and NK T cells (Pang, Narendran, 2008). Surface expression of both receptors is absent from memory or naive CD4⁺ or CD8⁺ T cells. We therefore speculated that treatment of adiponectin was not directly acting on T cells to mediate their migration. Instead, we hypothesised that the effects of adiponectin on T cells were mediated through one of these subsets (i.e. B cells; NK cells or NK T cells)

In this chapter, we aimed to identify the mechanism of action underlying the inhibition of T cell trans-endothelial migration by adiponectin, in particular we examine the potential involvement of B and NK cells in this process.

2. Results

2.1. Adiponectin does not modulate integrins expression on PBL or their capacity to migrate towards chemokines

Here we aimed to understand how adiponectin modulated PBL transmigration. First, we checked that the inhibition of PBL transmigration by adiponectin was not simply a reflection of cell death or apoptosis induced by this agent. This was assessed using propidium iodide (PI) exclusion assay, which stains dead cells, as it can only enter the cell through permeabilised membranes. We observed no difference in frequency of PI positive cells in presence of adiponectin compared to the untreated PBL (**Figure 6-1a**). We went on to measure the expression of Caspase-3 in PBL in order to verify the effects of adiponectin treatment were through induction of PBL apoptosis. Neutrophils (as positive control) were cultured overnight, a process which induces a high rate of apoptosis. Active caspase-3

Chapter 6- The adiponectin-dependent inhibition of T cell migration is mediated by B cells expression was detected in almost all neutrophils, indicating the high rate of apoptosis. In contrast, we did not detect the expression of active caspase-3 in freshly isolated PBL and we observed no effects of adiponectin treatment (**Figure 6-1b**).

Next we investigated whether adiponectin could modulate the expression of integrins and chemokine receptors on PBL, based on the supposition that these might modify the rate of PBL migration. We measured levels of $\alpha_L\beta_2$ (LFA-1), $\alpha_4\beta_1$ (VLA-4), CXCR3 (receptor to CXCL9, 10, 11) and DP-2 (receptor to prostaglandin-D2) by flow cytometry after one hour treatment with adiponectin. We did not identify any significant changes in the frequencies of $\alpha_L\beta_2$, $\alpha_4\beta_1$, CXCR3 and DP-2 positive cells, nor in the surface expression of these molecules on PBL treated with adiponectin compared to the untreated controls (**Figure 6-2**).

As no differences were found in the expression levels of CXCR3 and DP-2 receptors, we decided to analyse the migration of PBL treated with adiponectin towards their counter ligands, CXCL10 and PGD₂, which are specifically involved in the transmigration process of PBL (Ahmed *et al.*, 2011) For this purpose we set-up a chemotaxis assay using the Boyden chamber. The system is composed of two chambers separated by a filter with 8 μ m pores. The chemotactic agents are added to the bottom chamber and the PBL are placed in the top chamber. The number of cells that migrated through the filter was counted by acquisition of cell counts from the whole well volume on the flow cytometer. This assay revealed a strong migration of PBL towards SDF-1 α , used as a positive control. Chemotaxis of PBL towards CXCL10 and PGD₂ was significantly lower in comparison to the positive control. Importantly, we found no significant differences in chemotaxis towards SDF, CXCL10 or PGD-2 when cells were treated with adiponectin (**Figure 6-3**).

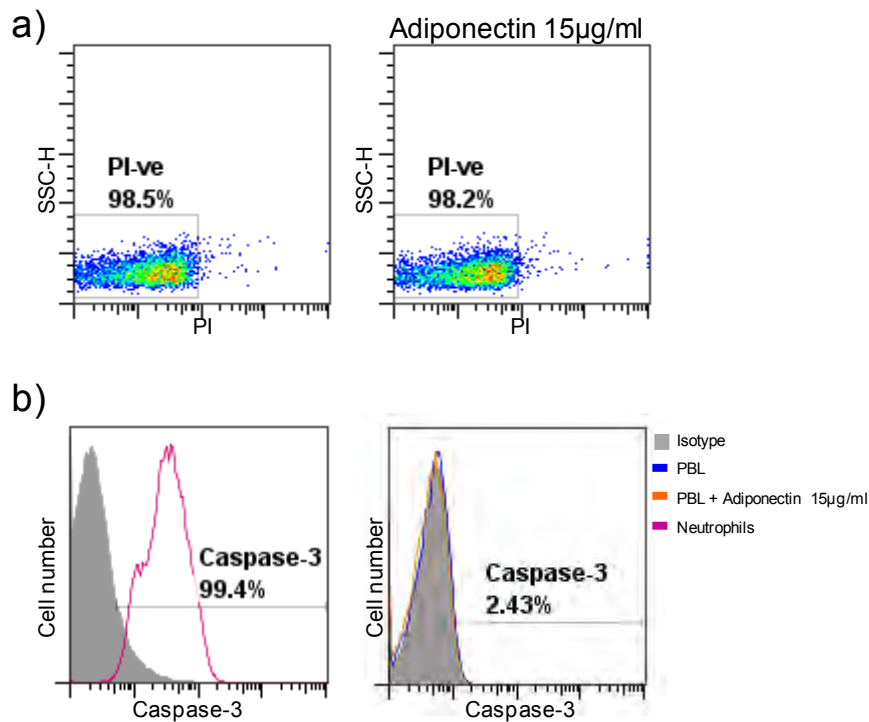


Figure 6-1: Adiponectin treatment does not cause PBL apoptosis and death

PBL and neutrophils were isolated from blood, (a) stained with PI to measure cell death and for (b) Caspase-3 to quantify cell apoptosis. Neutrophils were cultured over night at 37°C and stained the day after for Caspase-3. This induced high level of caspase-3 staining (left histograms) as expected. This was not observed for PBL treated with adiponectin for one hour (right histograms). The data are representative of three independent experiments for the PI staining and two for the Caspase-3 labelling.

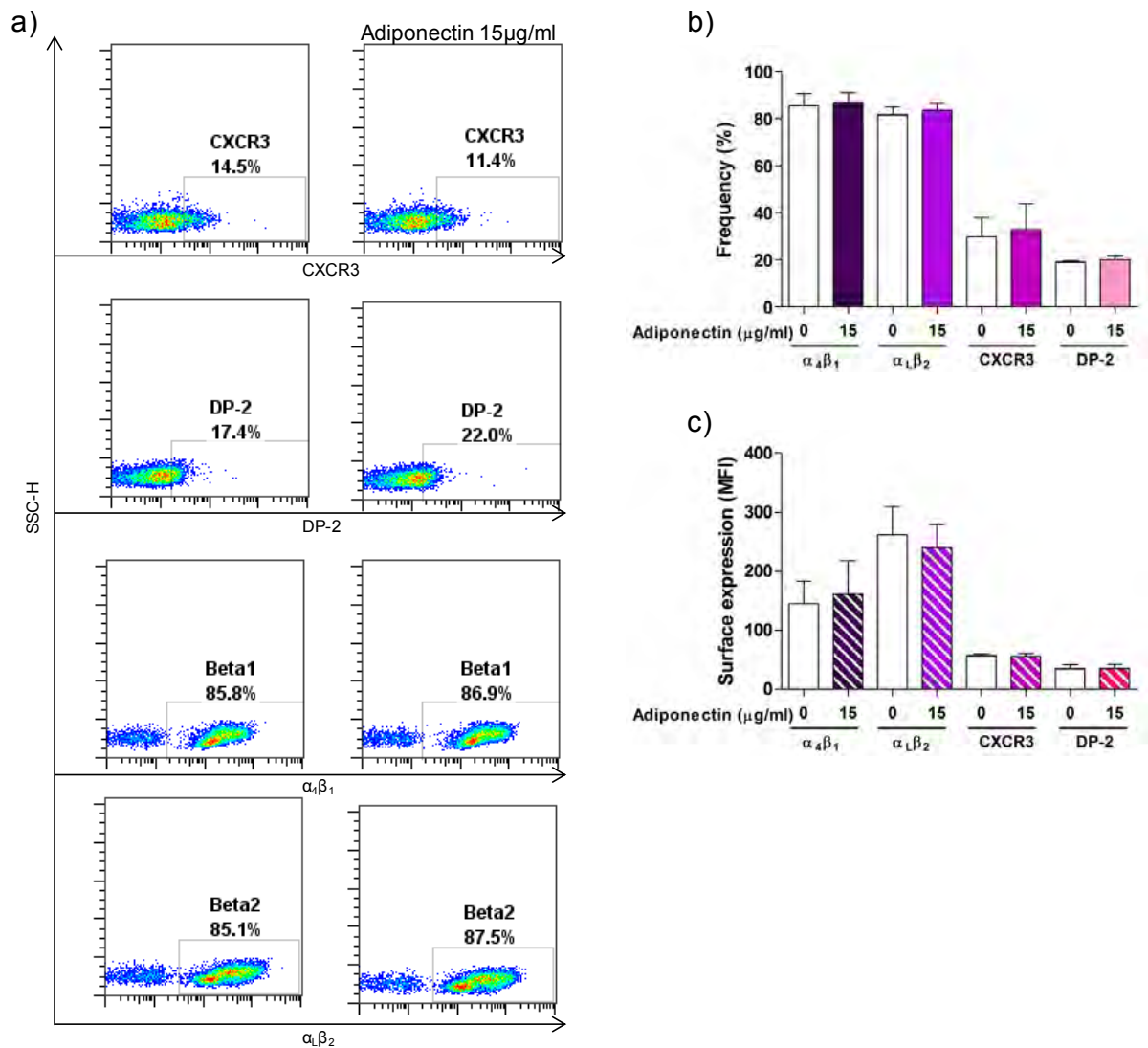


Figure 6-2: Expression of integrins and chemokine receptors is not altered by adiponectin treatment

PBL were isolated from the blood, stained for CXCR3, DP-2, $\alpha_4\beta_1$ (VLA-4) and $\alpha_L\beta_2$ (LFA-1) and analysed by flow cytometry. (a) Representative dots plots for the expression of each molecule are shown. Gates were set on the isotype controls and there is no difference in absence or presence of treatment with adiponectin for one hour on (b) frequency and (c) surface expression. Data are shown as mean \pm SEM and are a pool of three independent experiments. Data were analysed using one-way ANOVA and Bonferroni's multiple comparisons post-test. No significant differences were found.

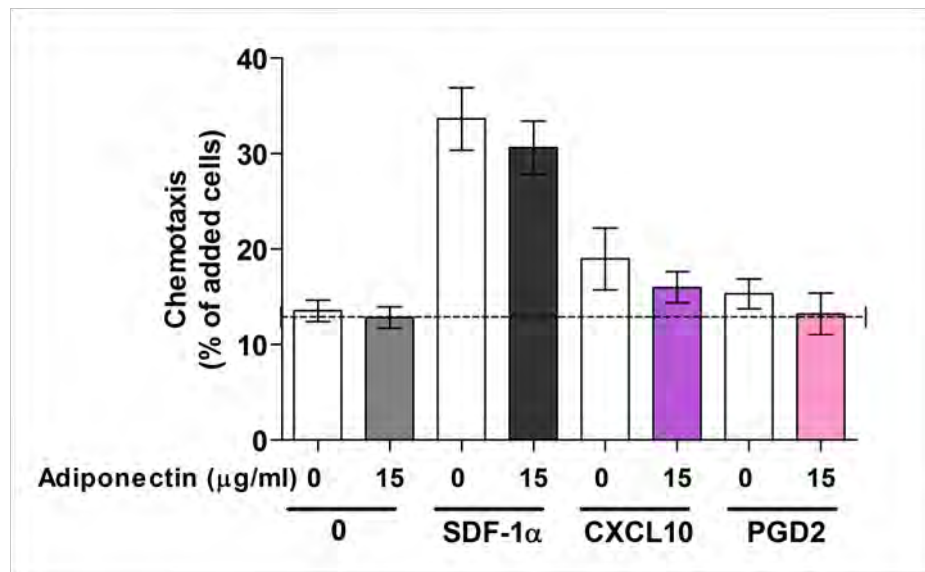


Figure 6-3: Adiponectin does not influence PBL chemotaxis

PBL were isolated from peripheral blood and incubated for one hour in presence or absence of adiponectin at 15μg/ml. After washes PBL were added to the top of the Boyden chamber. The bottom well contained media with SDF-1α (250ng/ml), CXCL10 (20ng/ml), PGD2 (1nM) or nothing. After 4 hours at 37°C, the total volume of the bottom well was acquired by flow cytometry to count the number of PBL that have migrated towards the chemokines. Data are shown as mean±SEM and are a pool of six donors. No significant differences were found between adiponectin treated and untreated PBL using one-way ANOVA and Bonferroni's multiple comparisons post-test.

2.2. Adiponectin-mediated inhibition of PBL transmigration is lost upon depletion of B cells.

We and others have observed that T cells do not express AR1 and AR2 expression on the cell surface (Pang, Narendran, 2008). Thus we assumed that the effects of adiponectin on T cell migration were mediated through another subset of PBL which did bare the receptors. We selectively removed B cells from the PBL population and analysed the effect of adiponectin under these conditions. We also looked at the effect of adiponectin on NK cells as they also express high levels of the receptors.

B cells were selectively removed from the PBL using anti-CD19 microbeads. This technique led to almost complete removal of B cells in the PBL pool, with on average only 0.7% of B cells remaining in the B cell negative PBL (Bs-ve PBL) (**Figure 6-4b**). B cells were also negatively sorted for the assay, where B cells were treated for one hour with adiponectin and added back to the B cell depleted PBL preparations (Bs reconstitution). This technique of magnetic depletion yielded B cells with an average purity of 98% (**Figure 6-4c**). Similarly, NK cells were positively purified using anti-CD56 microbeads. This also achieved high purity (91%) (**Figure 6-4d**). We chose not to sort T cells using anti-CD3 microbeads, because in our hands this induced T cell activation and consequently modified T cell behaviour in our assays (**Figure 6-5**). Indeed, anti-CD3 positively selected T cells aggregate and do not transmigrate efficiently across the endothelium.

Using these tools we investigated the effect of removing B cells from the PBL. Bs-ve PBL fraction was treated with adiponectin for one hour and transmigration of the remaining cells was assayed in the usual fashion. We found that eliminating B cells from the PBL abrogated the adiponectin-mediated inhibition of PBL transmigration (**Figure 6-6a**). The addition of adiponectin stimulated B cells back into the B cell depleted preparations (1:10 ratio) restored the inhibition of PBL transmigration by adiponectin (**Figure 6-6a**).

Finally, we checked whether adiponectin could also affect NK cells. The whole population of CD56⁺ NK cells were positively sorted and treated with adiponectin for one hour. This population includes the three subsets of NK cells (identified in Chapter V). NK cells exhibited efficient migration across the endothelium but adiponectin was not able to reduce their transmigration (**Figure 6-6b**). Similarly, B cells treated with adiponectin and added to the NK cells fraction (Bs+ NKs) at 1:10 ratio did not induce inhibition of NK cell transmigration. These results indicate that the effect of B cells is specific for T cells. Moreover it transpires that the effects on T cells are probably specific to B cells, as NK cells treated with adiponectin and reconstituted with T cells (1:10), did not reduce T cell migration.

All together, these results indicate that the inhibition of PBL transmigration by adiponectin is mediated by B cells. These studies were also repeated under flow conditions (**Figure 6-7**). The Bs reconstitution data in these conditions did not reach significance, but the p-value is very close to 0.05 which suggests a trend to restoration of the adiponectin effect when B cells are added back to Bs-ve PBL.

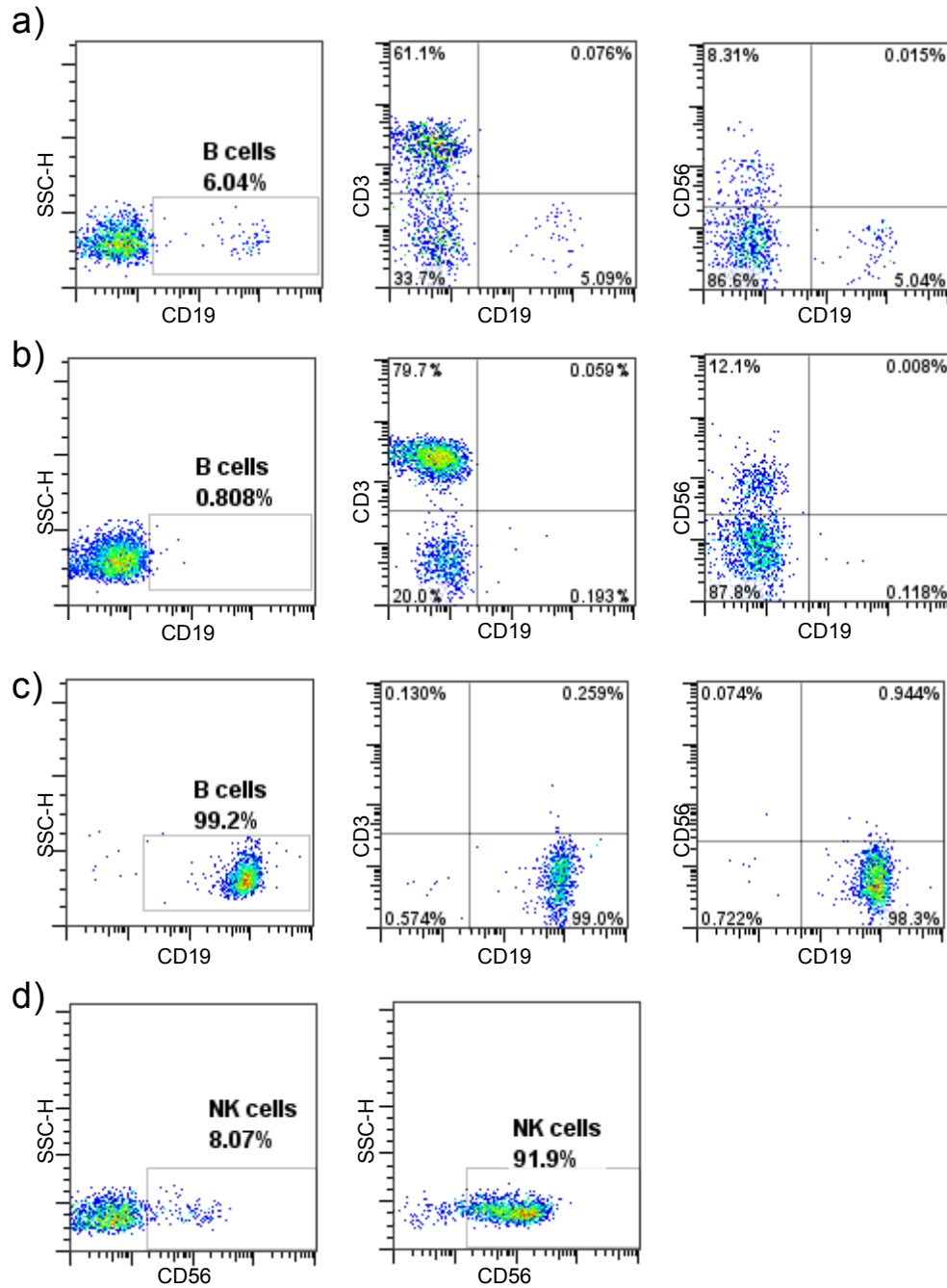


Figure 6-4: Representative dot plots of B cell depletion and NK cells selection

(a) Whole PBL were isolated from peripheral blood stained for B cell, T cell and NK cells markers. (b) PBL were depleted for B cells by anti-CD19 positive selection which removed almost all B cells giving the Bs-ve PBL fraction. (c) B cells were also negatively purified with 99.2% purity and used for reconstitution of Bs-ve PBL fraction referred as Bs reconstitution fraction. (d) NK cells were positively selected for CD56 leading to 91.9% purity. The data are representative of at least four independent experiments

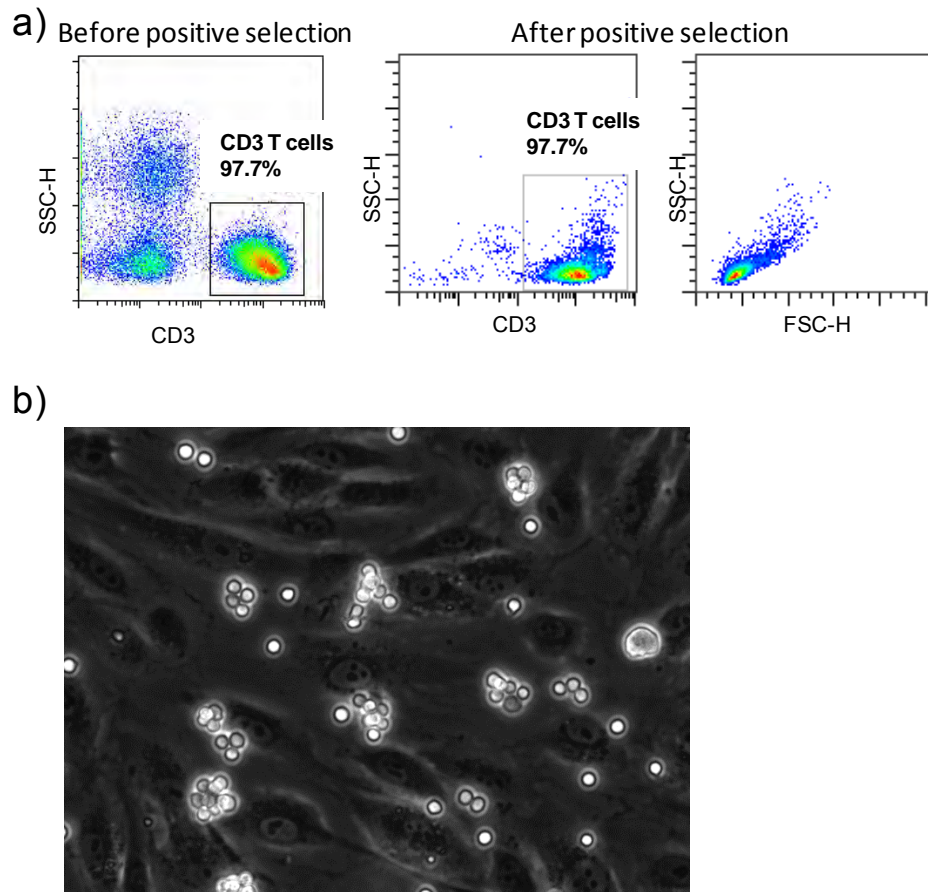


Figure 6-5: Anti-CD3 positive selection of T cells triggers cell activation and changes in the ability to migrate across endothelial cells

CD3⁺ T cells were isolated from peripheral blood using anti-CD3 magnetic selection. (a) This technique led to 97.7% purity (left) but caused T cell activation as observed by the increase in granularity and size on the forward/side scatter dot plot (right). (b) Stimulation of T cells by anti-CD3 prompt T cells to form small aggregates on the top on the endothelium, which is typical of T cell stimulation *in vitro*. This alters the transmigration capacity of this population and therefore ruled out anti-CD3 positive selection for the future experiments. The data are representative of two independent experiments.

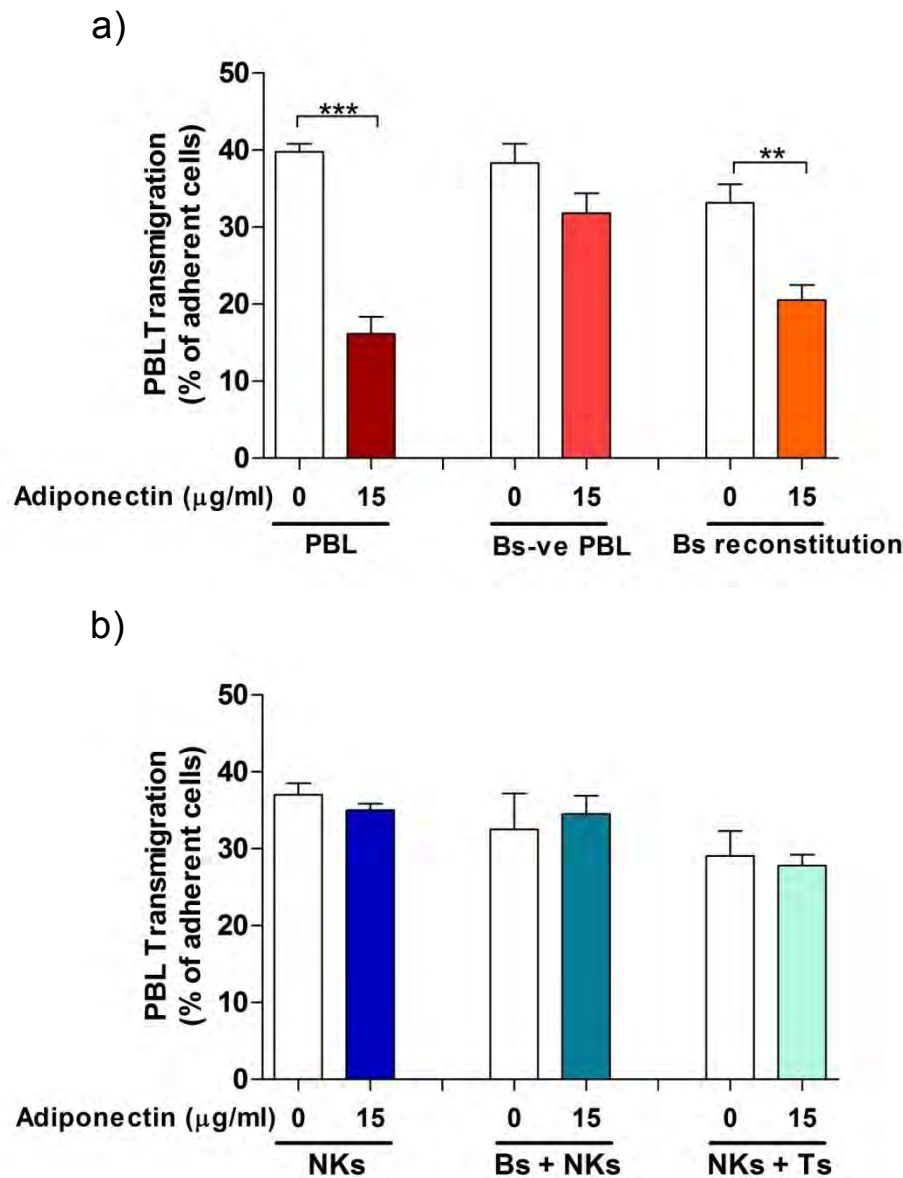


Figure 6-6: B cells mediate the adiponectin-induced inhibition of PBL transmigration

(a) PBL were isolated from peripheral blood and transmigration assays were performed using TNF- α /IFN- γ stimulated HUVEC and adiponectin pre-treated whole PBL, B cell depleted PBL (Bs-ve PBL), or B cell depleted PBL to which B cells had been re-constituted (Bs reconstitution) at 1 to 10 ratio. (b) Transmigration was also measured for NK cells (NKs), NK cells reconstituted with adiponectin-treated B cells (Bs+NKs) at 1 to 10 ratio, and for T cells reconstituted with adiponectin-treated NK cells (NKs+Ts) at 1 to 10 ratio. Data are shown as mean \pm SEM and are a pool of at least three independent experiments. Data were analysed using one-way ANOVA and Bonferroni's multiple comparisons post-test. ** $p \leq 0.01$, *** $p \leq 0.001$.

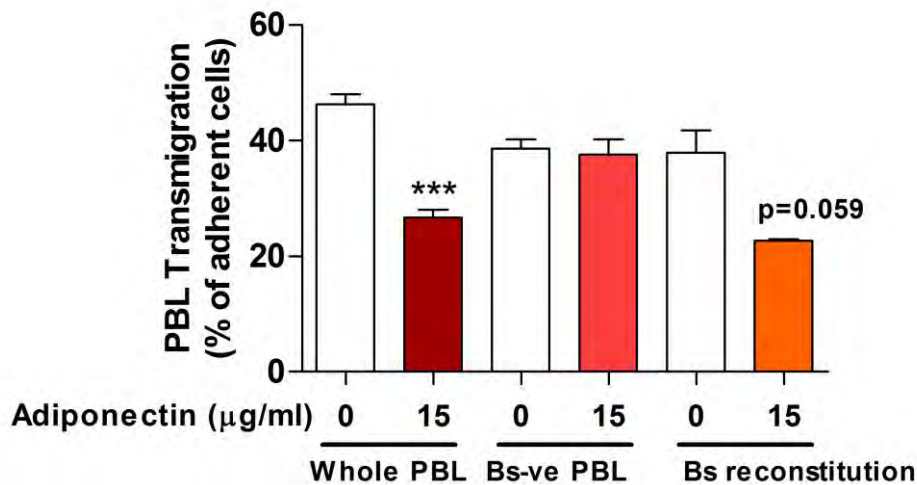


Figure 6-7: B cells mediate the adiponectin-induced inhibition of PBL transmigration in flow conditions

PBL were isolated from peripheral blood and transmigration assays in flow conditions were performed using TNF- α /IFN- γ stimulated HUVEC and adiponectin pre-treated B cell depleted PBL (Bs-ve PBL), or B cell depleted PBL to which B cells had been re-constituted (Bs reconstitution) at 1 to 10 ratio. No significant differences between adiponectin treated and untreated cells were found, but a trend was observed when B cells were added back to the PBL fraction. Data are shown as mean \pm SEM and are a pool of two independent experiments. Data were analysed using one-way ANOVA and Bonferroni's multiple comparisons post-test. ***p \leq 0.001.

2.3. B cells are recruited to the endothelium surface but do not transmigrate

Previous findings using transwell system show that the predominant subsets of PBL that migrate across the endothelium are CD4⁺ memory T cells followed by CD8⁺ memory T cells (McGettrick *et al.*, 2009). However, there are no data available on the behaviour of PBL subpopulations in our *in vitro* system, especially on B cells and NK cells.

Here we aimed to identify the phenotype of PBL subsets that are firmly adhered to the endothelium (phase bright) and that have migrated across the HUVEC (phase dark). PBL were added to HUVEC that had been cultured in low serum media and stimulated with TNF- α /IFN- γ for 24 hours. After removal of none-adherent cells, firmly adherent cells were harvested from the surface of the endothelium with EDTA and gentle agitation. Migrated cells were harvested along with the HUVEC monolayer using accutase treatment for 30 seconds. Both fractions were labelled for the B cells, CD4/CD8 memory/naive T cells, NK T cells, NK cells and CD56^{high} NK cells subsets and analysed by flow cytometry. Under these conditions, we observed preferential recruitment of CD4⁺ memory T cells in both the firmly adherent and transmigrated populations (**Figure 6-8 and 9**). On the top of the endothelium, the percentage of CD4⁺ memory T cells was the highest followed by CD8⁺ memory T cells (**Figure 6-9a**). There was also recruitment of B cells, NK cells and CD56^{high} NK cells. The main subsets that had transmigrated were CD4⁺ memory T cells, closely followed by NK T cells, naive CD8⁺ T cells and NK cells (**Figure 6-9b**). Very few B cells, CD4⁺ naive T cells and CD56^{high} NK cells were found underneath the endothelium.

We then calculated the enrichment ratio on the surface and underneath the endothelium by dividing the number of cells firmly adhered or migrated for each subset by the number of cells added (based on whole PBL staining). This analysis generated slightly different results. On the top of the endothelium, we found enrichment for B cells, memory CD4⁺ and CD8⁺ T cells, followed by CD56^{high} NK cells, NK T cells and naive CD8⁺ T cells.

Enrichment for naive CD4⁺ and NK cells was low on the endothelium surface (**Figure 6-9c**). Enrichment of different subsets in the transmigrated pool showed that NK cells were most efficiently enriched, closely followed by NK T cells, CD4⁺ memory T cells and slightly less memory and naive CD8⁺ T cells and CD56^{high} NK cells (**Figure 6-9d**). As before, there is negligible migration of B cells and naive CD4⁺ T cells.

These experiments were repeated in flow conditions. Using the flow-based assay, firmly adhered PBL were removed by perfusing EDTA across the HUVEC and collecting them in a plastic syringe on the Harvard pump. Transmigrated PBL were again collected by accutase treatment. Whole PBL (blood) and both firmly adhered and transmigrated fractions were analysed for PBL subpopulations by flow cytometry (**Figure 6-10a-d**). Using this approach, we obtained similar data to those found under static conditions with the exceptions that there was a higher percentage of naive CD8⁺T cells and NK cells and fewer CD8⁺ memory T cells (**Figure 6-10e, g**). If we consider enrichment ratio for the top fraction, there are no major differences between all the PBL subpopulations compared to static adhesion assays. The enrichment ratio for the transmigrated cells also shows a similar profile to static conditions (**Figure 6-10f, h**). In conclusion, these data indicate an ability of B cells to bind to the cytokine stimulated endothelium; however, in the conditions we used, B cells were not able to transmigrate efficiently. Therefore, it is important to understand which signals allow their surface adhesion.

We went on to examine the functional basis of B cell adhesion. Antibodies against $\alpha_4\beta_1$ (Max68P), $\alpha_L\beta_2$ (IB4) and VCAM-1 (4B2+GH12) were used to block adhesion. We observed a strong inhibition of B cell adhesion when $\alpha_4\beta_1$ (VLA-4) and VCAM-1 were blocked (**Figure 6-11**). Furthermore, we looked at blockade of chemokine receptor signalling using pertussin toxin (PTx). However, inhibition of GPCR signalling using this agent did not cause a loss of B cell adhesion. These results indicates that B cell adhesion is mediated by

$\alpha_4\beta_1$ (VLA-4)/VCAM-1 interaction, although the activating stimulus which stabilises B cell adhesion on the surface remains unknown.

Finally, we sought to determine which subsets of B cells are preferentially recruited to the endothelium. Indeed, multiple B cells subpopulations exist, and to our knowledge, no study has explored which B cell subsets are preferentially recruited to cytokine stimulated endothelium. We used the same technique previously described to harvest the PBL from the endothelium surface and labelled the cells with CD19, CD38, IgD, IgM and CD27. This staining allowed us to differentiate between naive (mature) B cells (1) (identified as $CD38^+IgD^+IgM^+$), memory B cells (2) ($CD38^-IgD^-IgM^+CD27^+$), plasma cells (3) ($CD38^{+++}IgD^-IgM^+CD27^-$), intermediate B cells (4) ($CD38^{++}IgD^+IgM^+$) and transitional B cells (5) ($CD38^{+++}IgD^+IgM^+$) (**Figure 6-12a, b**). We calculated the enrichment ratio as previously described by dividing the percentage of each B cell subset on the endothelium surface by the percentage of each B cell subpopulations in whole blood. Using this method of analysis, we found a trend towards enrichment of plasma cells and a modest enrichment of memory B cells (**Figure 6-12c**). These two B cell subsets constitute the effector B cell populations, as they are generated after encounter with an antigen.

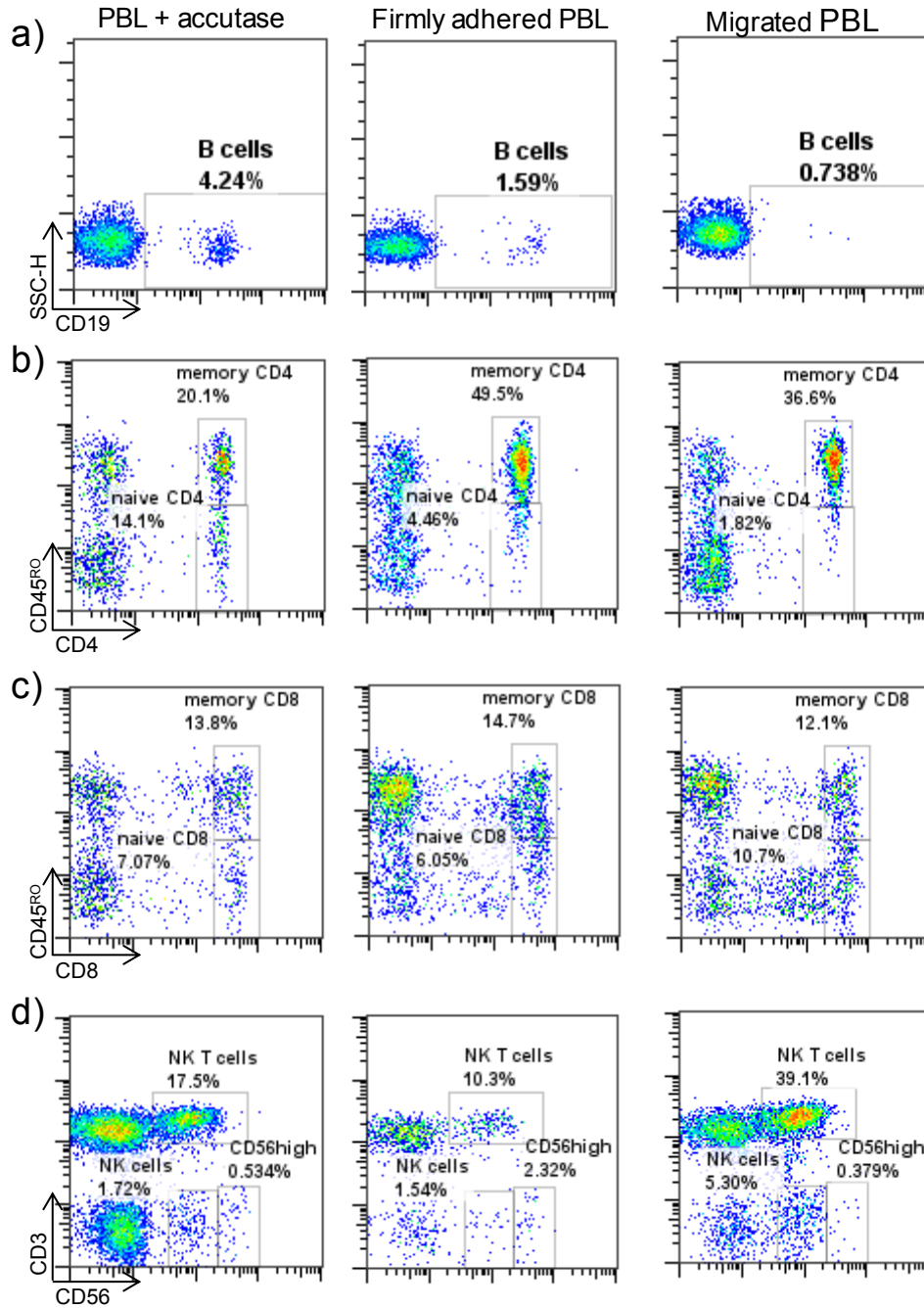


Figure 6-8: Representative dot plots of transmigrated and firmly adhered PBL subsets on the endothelium in static conditions

PBL were isolated from peripheral blood and static transmigration assays were performed using TNF- α /IFN- γ stimulated HUVEC as previously described. After acquisition, PBL firmly adhered to endothelium were removed using EDTA and gentle agitation. The HUVEC and the migrated PBL were collected using accutase. Whole PBL treated with accutase were used to determine the proportion of each subset added. Whole accutase treated PBL, firmly adhered and transmigrated fractions were stained and analysed by flow cytometry for (a) B cells, (b) CD4 memory and naive T cells, (c) CD8 memory and naive T cells and (d) NK cells subsets. The data are representative of at least three independent experiments.

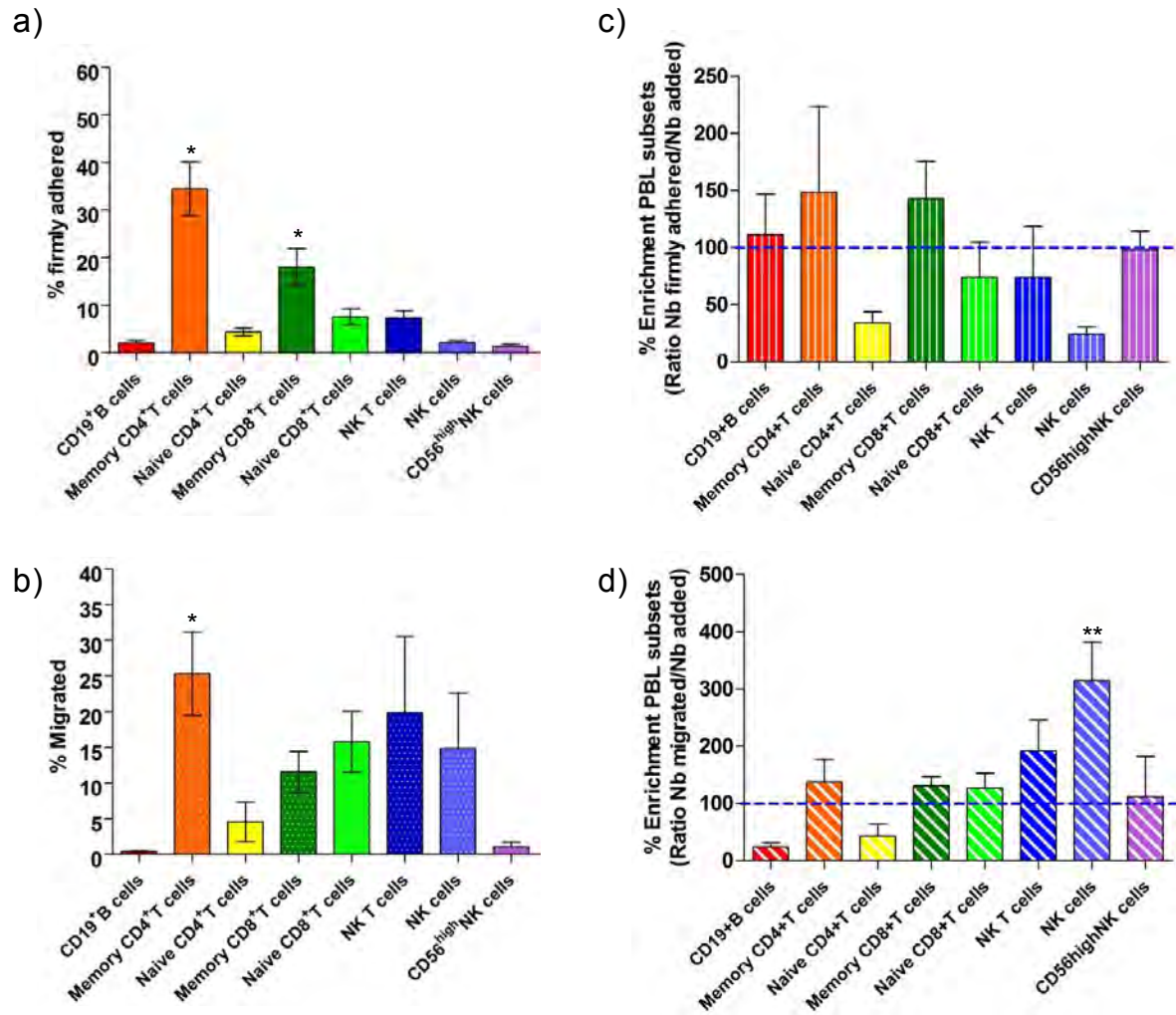


Figure 6-9: Behaviour of firmly adhered and migrated PBL subsets in static conditions
PBL were isolated from peripheral blood and static transmigration assays were performed using TNF- α /IFN- γ stimulated HUVEC and cells were stained as previously described on Figure 6.9. (a) The percentages of each subset on the top of HUVEC and (b) transmigrated were determined by flow cytometry. The enrichment ratio for each subset was calculated by dividing the percentage of (c) cells firmly adhered or (d) transmigrated by the percentage of each subset added determined on the whole accutase treated PBL fraction. Data are shown as mean \pm SEM and are representative of at least three independent experiments. Data were analysed using one-way ANOVA and Bonferroni's multiple comparisons post-test. * $p \leq 0.05$, ** $p \leq 0.01$.

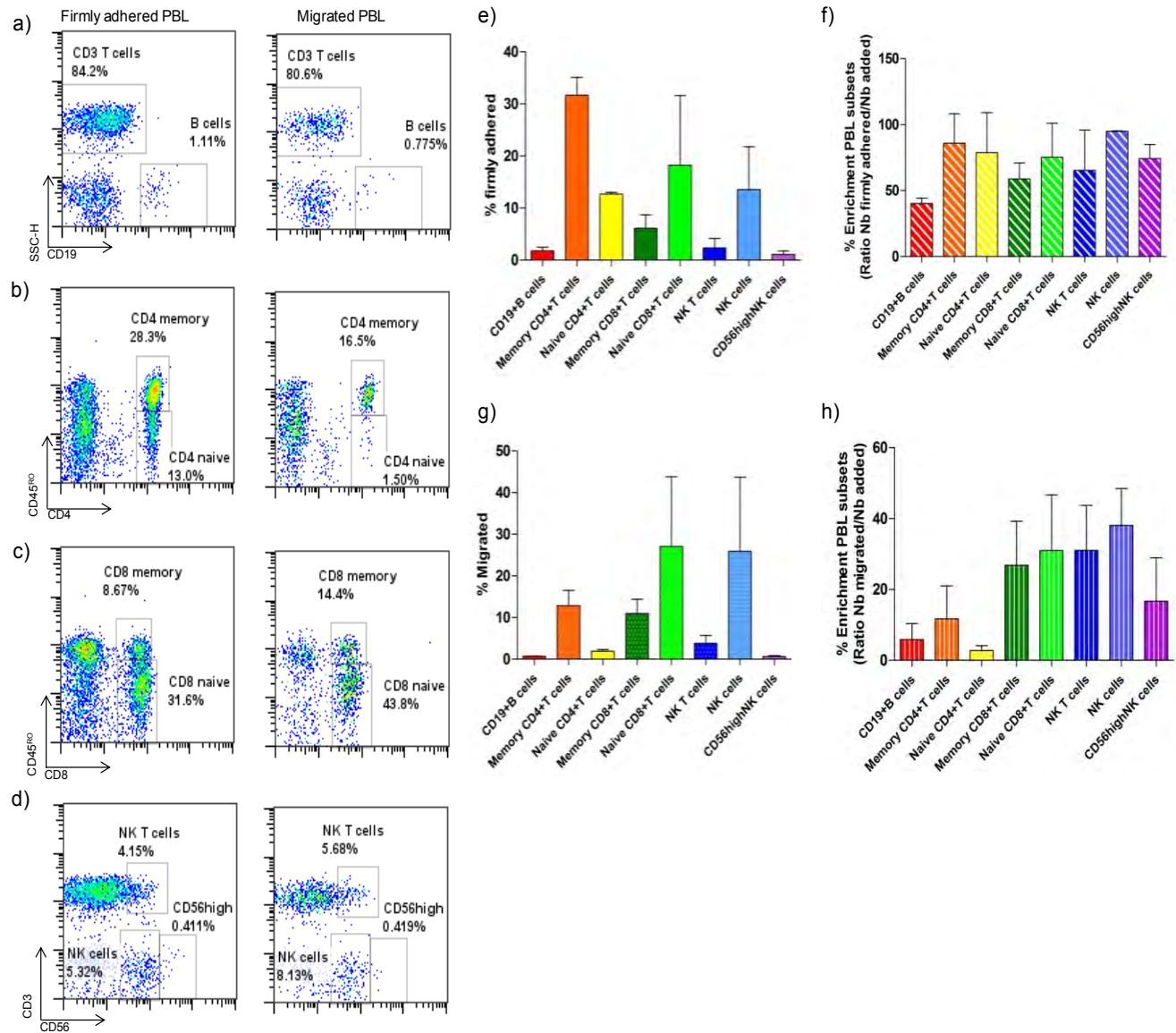


Figure 6-10: Behaviour of firmly adhered and migrated PBL subsets in flow conditions
PBL were isolated from peripheral blood and transmigration flow assays were performed using TNF- α /IFN- γ stimulated HUVEC as previously described. After acquisition, PBL firmly adhered to endothelium were removed using EDTA under flow conditions. The HUVEC and the migrated PBL were collected using accutase. Whole accutase treated PBL, firmly adhered and transmigrated fractions were stained and analysed by flow cytometry for (a) B cells, (b) CD4⁺ memory and naive T cells, (c) CD8 memory and naive T cells and (d) NK cell subsets. (e) The percentage of each subset on the top of HUVEC and (g) transmigrated were determined by flow cytometry. The data are representative of at least three independent experiments. The enrichment ratio for each subset was calculated by dividing the percentage of (f) cells firmly adhered or (h) transmigrated by the percentage of each subset added determined on the whole accutase treated PBL fraction. Data are shown as mean \pm SEM and are representative of at least three independent experiments. Data were analysed using one-way ANOVA and Bonferroni's multiple comparisons post-test.

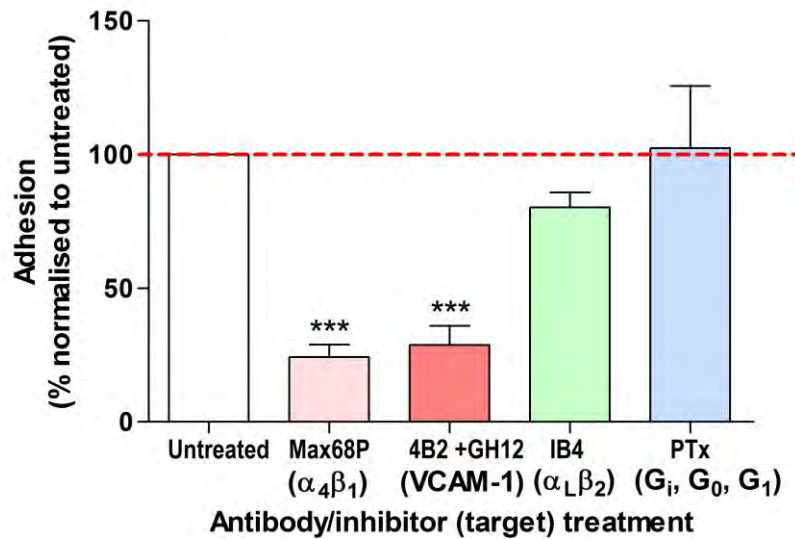


Figure 6-11: B cell adhesion is mediated by VLA-4 ($\alpha_4\beta_1$) -VCAM interaction

B cells were negatively selected and recruitment was measured on HUVEC stimulated with TNF- α and IFN- γ . B cells were pre-treated with antibodies selectively blocking $\alpha_4\beta_1$ (Max68P) and $\alpha_L\beta_2$ (IB4) integrins binding to their respective VCAM-1 and ICAM-1 adhesion molecules. HUVEC were treated with 4B2 and GH12 to inhibit VCAM-1 interactions. In addition GPCR signalling blockade was realised using Pertussin toxin (PTx) known to stop transmigration mediated by chemokines receptors signalling. Data are represented as mean \pm SEM and represent three independent experiments. Data were analysed using one-way ANOVA and Dunnet's multiple comparisons post-test. *** $p \leq 0.001$. **Courtesy of Dr Helen McGettrick.**

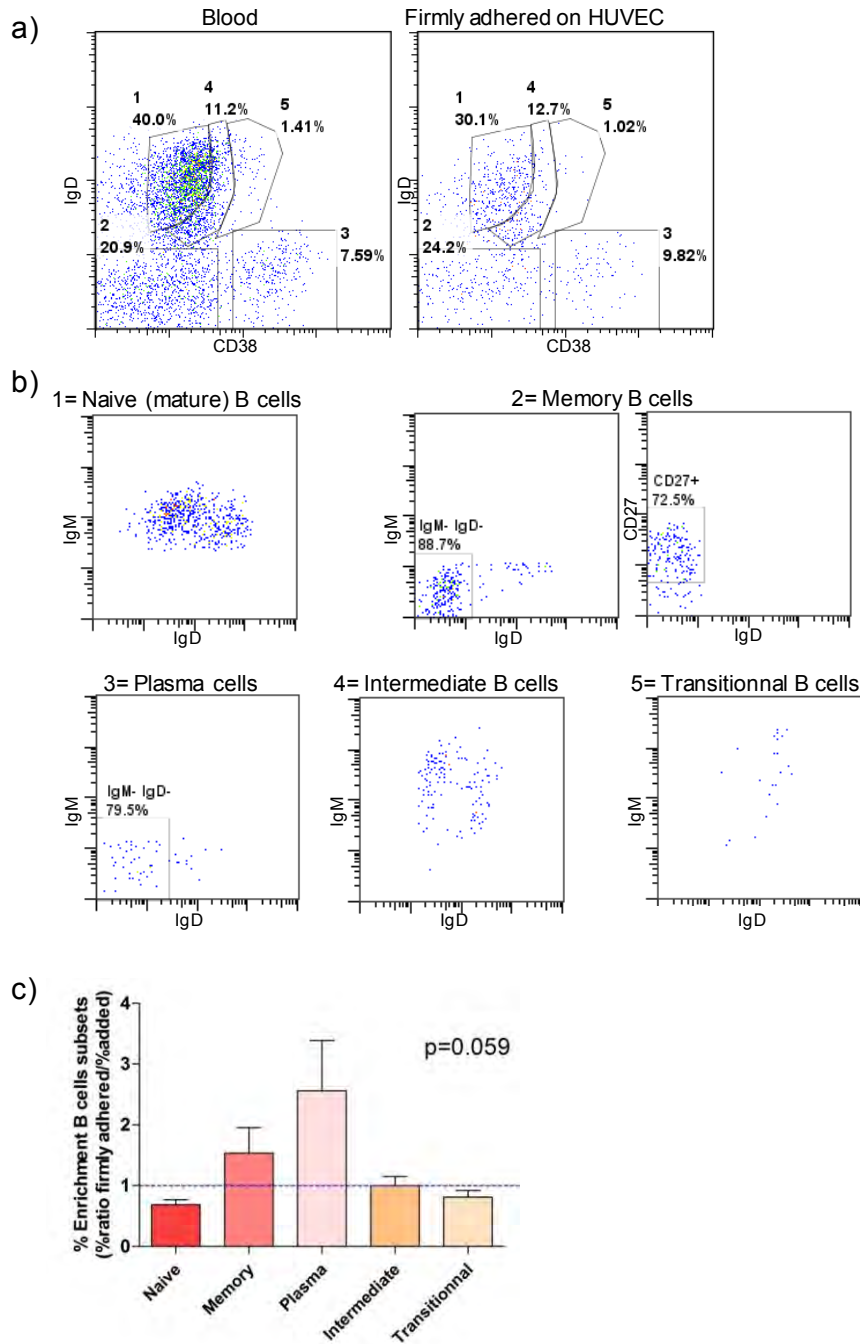


Figure 6-12: Preferential recruitment of effector B cells at the surface of the endothelium

PBL were isolated from peripheral blood and transmigration flow assays were performed using TNF- α /IFN- γ stimulated HUVEC as previously described. (a) After acquisition, PBL firmly adhered to endothelium were removed using EDTA plus gentle agitation and labelled for the different B cell subpopulations. (b) Naive (mature) B cells (1) were identified as CD38⁺ IgD⁺ IgM⁺, memory B cells (2) as CD38⁻ IgD⁻ IgM⁻ CD27⁺, plasma cells (3) as CD38⁺ IgD⁻ IgM⁻ CD27⁻, intermediate B cells (4) as CD38⁺ IgD⁺ IgM⁺ and transitional B cells (5) as CD38⁺ IgD⁺ IgM⁺. (c) The enrichment ratio on the endothelial surface was calculated by dividing the percentage of each subset recruited on HUVEC by the percentage of each subset added from the whole PBL (blood) staining. Data are shown as mean \pm SEM and are representative of three donors. Data were analysed using one-way ANOVA and Bonferroni's multiple comparisons post-test.

2.4. Peritoneal infiltration of lymphocytes is higher in B cell knock-out mice

We demonstrate that adiponectin-stimulated B cells modulate T cell migration *in vitro*. Here, we aimed to validate this effect *in vivo*. In collaboration with Prof. Chris Buckley and Dr Francesca Barone, we used a peritoneal mouse model of acute inflammation to study the effect of B cells on T cell migration *in vivo*. Peritoneal inflammation was induced by injection of 1 mg of zymozan A into the peritoneum of wild-type (wt) mice or B cell knock-out (KO) mice (Jh-/-), both on a BALB/c background. After 48 hours, peritoneal cells were isolated and counted by flow cytometry using CD3 expression for T cells, CD4⁺ and CD8⁺ T cells and their naive (CD62L⁺CD44⁻), central memory (CD62L⁺CD44⁺) and effector memory (CD62L⁻CD44⁺) subsets. We also, stained for CD11c⁺DCs and F4/80/podoplanin positive macrophages. The whole volume of each tube was acquired on the flow cytometer to allow accurate counting. Peritoneal and circulating leukocytes were gated based on forward/side scatter profile and pulse-width to exclude duplets of cells (**Figure 6-13**).

Interestingly, absence of B cells resulted in a significantly larger number of CD3⁺T cell in the inflamed peritoneum compared to the wt (**Figure 6-14**). The number of T cells was slightly higher in the peritoneum of B cell KO mice treated with PBS compared to the wt, but this difference was not significant. Injection of zymosan also induced a slight increase of T cell number in the peritoneum of the wt, but not as high as in the B cell KO. Further analysis of T cell sub-populations revealed that the high recruitment of T cells in the peritoneum of B cells KO mice is mainly composed of CD4⁺ T cells (**Figure 6-15**). Indeed the absolute number of CD4⁺ T cells was significantly higher in the inflamed peritoneum of B cell KO mice compared to the inflamed wt and to the B cell KO treated with PBS.

We went on to analyse the specific recruitment of CD4⁺ naive, central memory and effector memory T cells in the peritoneum. We observed a significantly higher recruitment of CD4⁺ effector memory T cells in the inflamed peritoneum of B cell KO mice compared to

Chapter 6- The adiponectin-dependent inhibition of T cell migration is mediated by B cells non-inflamed conditions. However, there was not a significant difference when compared with the inflamed wt (**Figure 6-16**). Regarding CD8⁺T cells subsets, no significant differences were found for effector memory T cells, however, there was a significant increase of naive CD8⁺ T cells number in the inflamed peritoneum of B cell KO mice compared to the inflamed wt (**Figure 6-17**). We also counted the number of macrophages and dendritic cells (DCs) in the peritoneum. This analysis revealed no significant differences between the inflamed or non-inflamed wt and B cell KO mice for both macrophages (**Figure 6-18**) and DCs (**Figure 6-19**).

Finally, the same staining protocols were conducted on circulating leukocytes. CD3⁺ T cells and B cells were found in the blood of wt with or without zymosan. CD3⁺ T cells but no B cells were found in the blood B cell KO mice (**Figure 6-20**). Additionally and as expected, we found very few macrophages and DCs in either wt or B cell KO with or without zymosan. This is in agreement with the usual tissue/lymphoid organs location of these cell types. CD4⁺ and CD8⁺ T cells were found in both wt and B cell KO mice, however, in both strains of mice, and with or without zymosan, naive T cells were predominantly found in the circulation (**Figure 6-21**). Very few central memory and effector memory CD4⁺ or CD8⁺ T cells were detected in the blood of these mice.

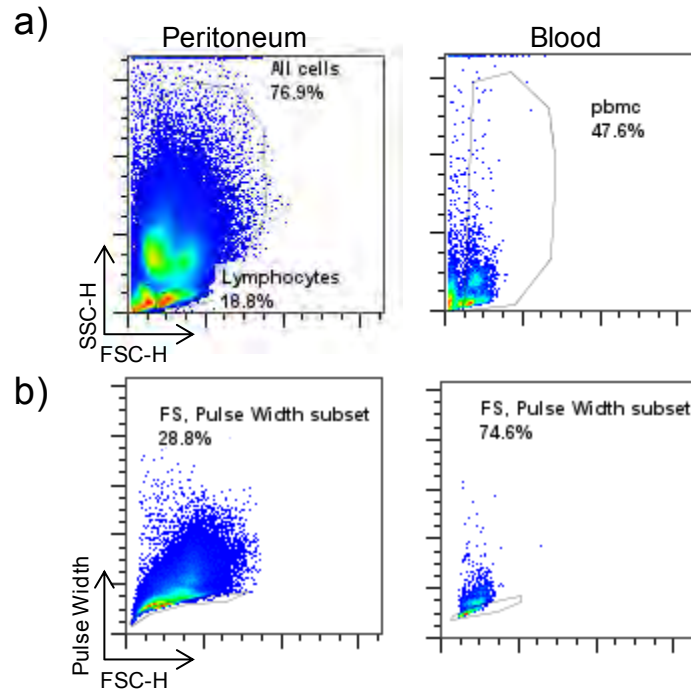


Figure 6-13: Gating strategy for leukocytes in the peritoneum and in the blood

Peritoneal lavage and cardiac puncture allowed collection of leukocytes present in the peritoneum and the blood respectively of wt and Bs KO mice with or without inflammation. After labelling cells were acquired by flow cytometry. (a) The whole peritoneal leukocyte population was gated based on forward/side scatter dot plots and (b) duplets of cells were excluded by gating on forward side scatter and pulse width.

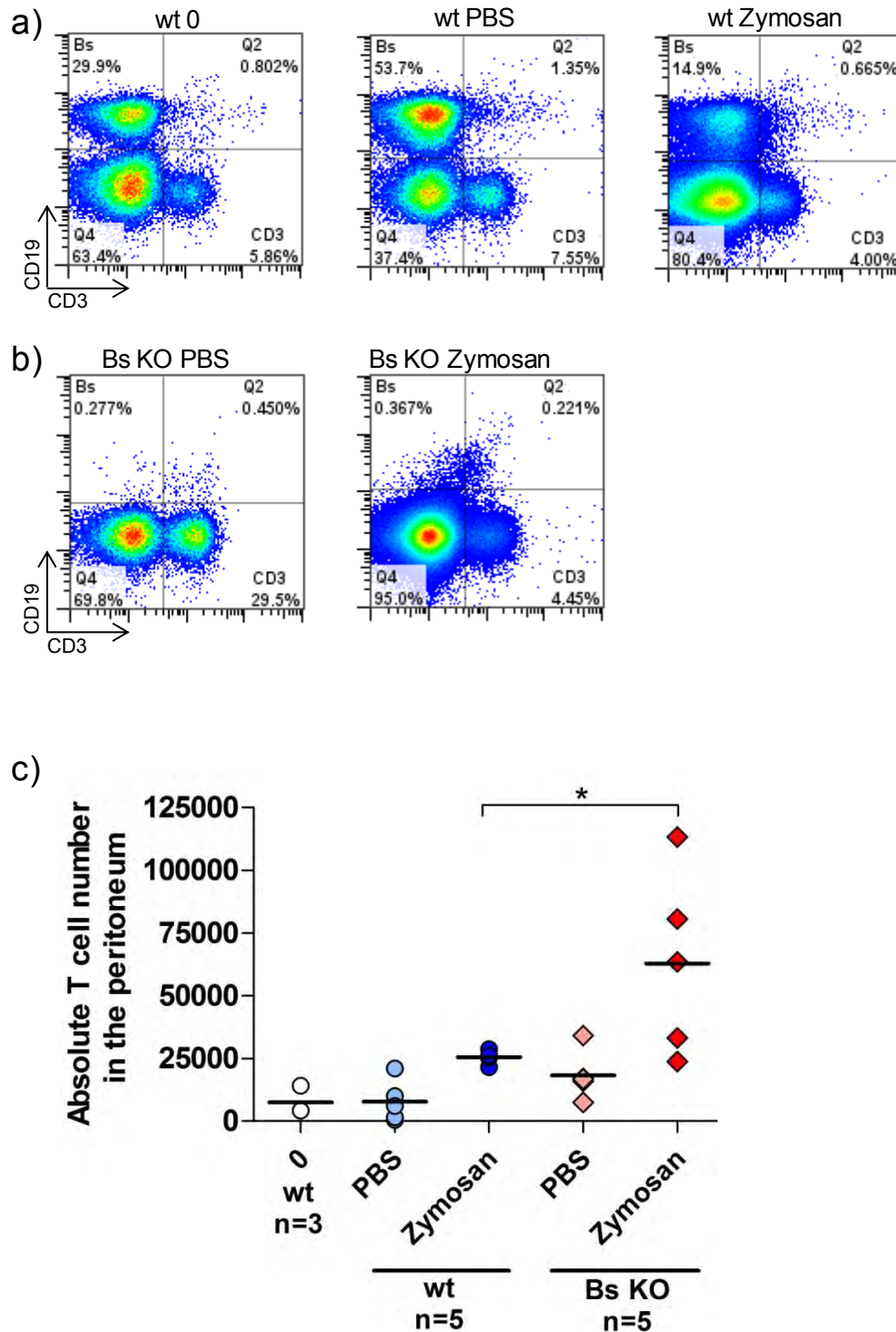


Figure 6-14: Absolute T cell number is higher in the inflamed peritoneum of B cell knock-out mice compared to the wt mice

Leukocytes were collected from the peritoneum after 48 hours without any injection (wt 0), with PBS injection (wt PBS, Bs KO PBS) and with zymosan injection (wt zymosan, Bs KO zymosan) in (a) wt and (b) Bs KO mice. T cells were identified by expression of CD3 and B cells using CD19 and B220. (c) Data for each mouse groups were pooled, represented as mean and were analysed using one-way ANOVA and Bonferroni's multiple comparisons post-test. * $p \leq 0.05$.

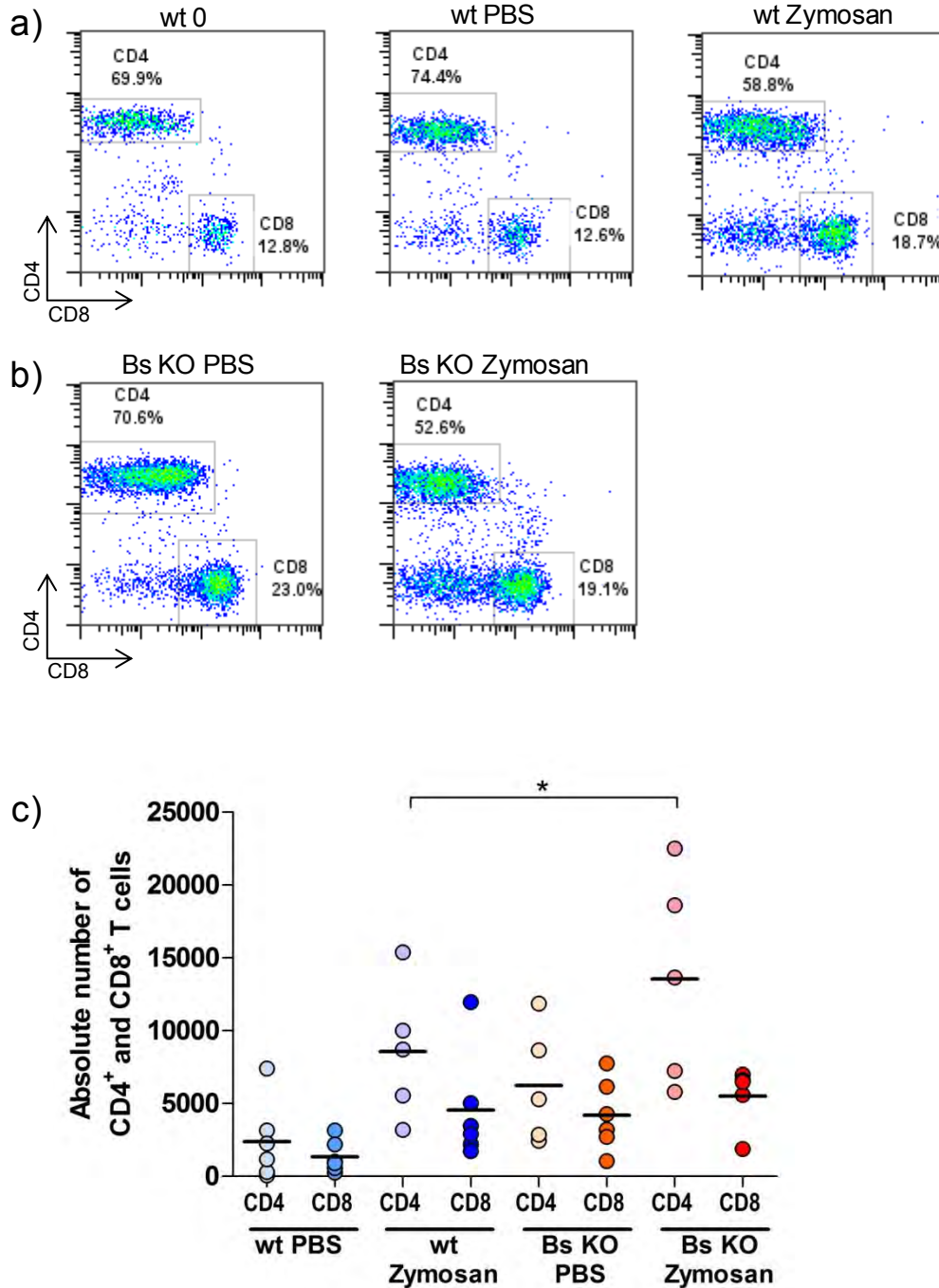


Figure 6-15: Absolute number of CD4⁺ T cells is higher in the inflamed peritoneum of B cell knock-out mice compared to the wt mice

Leukocytes were collected from the peritoneum after 48 hours without any injection (wt 0), with PBS injection (wt PBS, Bs KO PBS) and with zymosan injection (wt zymosan, Bs KO zymosan) in (a) wt and (b) Bs KO mice. CD4⁺ and CD8⁺ T cells were identified by gating on CD3 first and by the expression of CD4 and CD8. (c) Data for each mouse groups were pooled, represented as mean and were analysed using one-way ANOVA and Bonferroni's multiple comparisons post-test. *p ≤ 0.05.

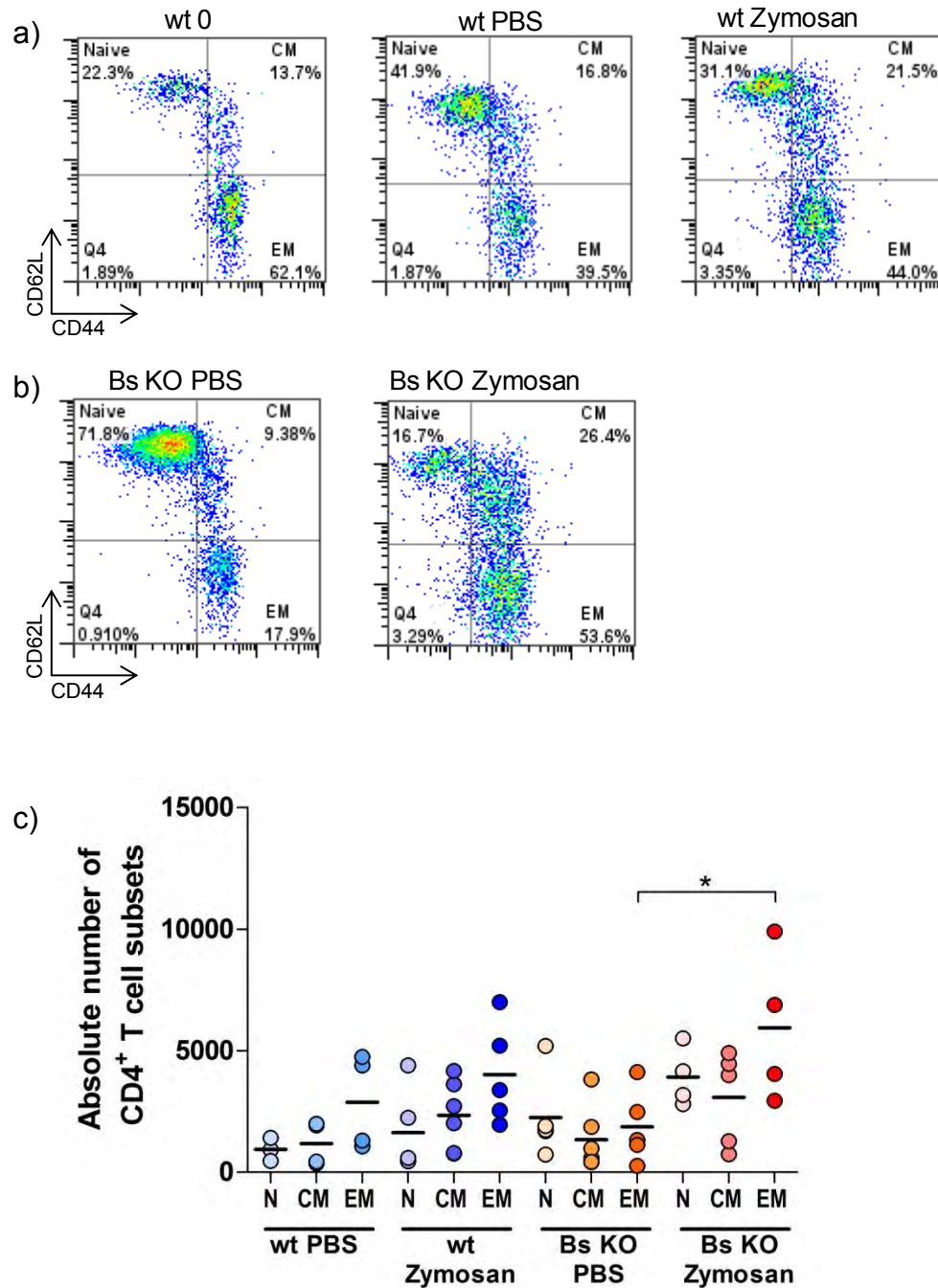


Figure 6-16: Absolute number of effector memory CD4⁺ T cells is slightly higher in the inflamed peritoneum of B cell knock-out mice compared to the wt mice

Leukocytes were collected from the peritoneum after 48 hours without any injection (wt 0), with PBS injection (wt PBS, Bs KO PBS) and with zymosan injection (wt zymosan, Bs KO zymosan) in (a) wt and (b) Bs KO mice. CD3⁺ and CD4⁺ T cells were first gated and naive, central memory and effector memory T cells were identified by the expression of CD44 and CD62L. Naive CD4 are CD62L⁺CD44⁻, central memory T cells are CD62L⁺CD44⁺ and effector memory T cells are CD62L⁻CD44⁺. (c) Data for each mouse groups were pooled, represented as mean and were analysed using one-way ANOVA and Bonferroni's multiple comparisons post-test. *p≤0.05.

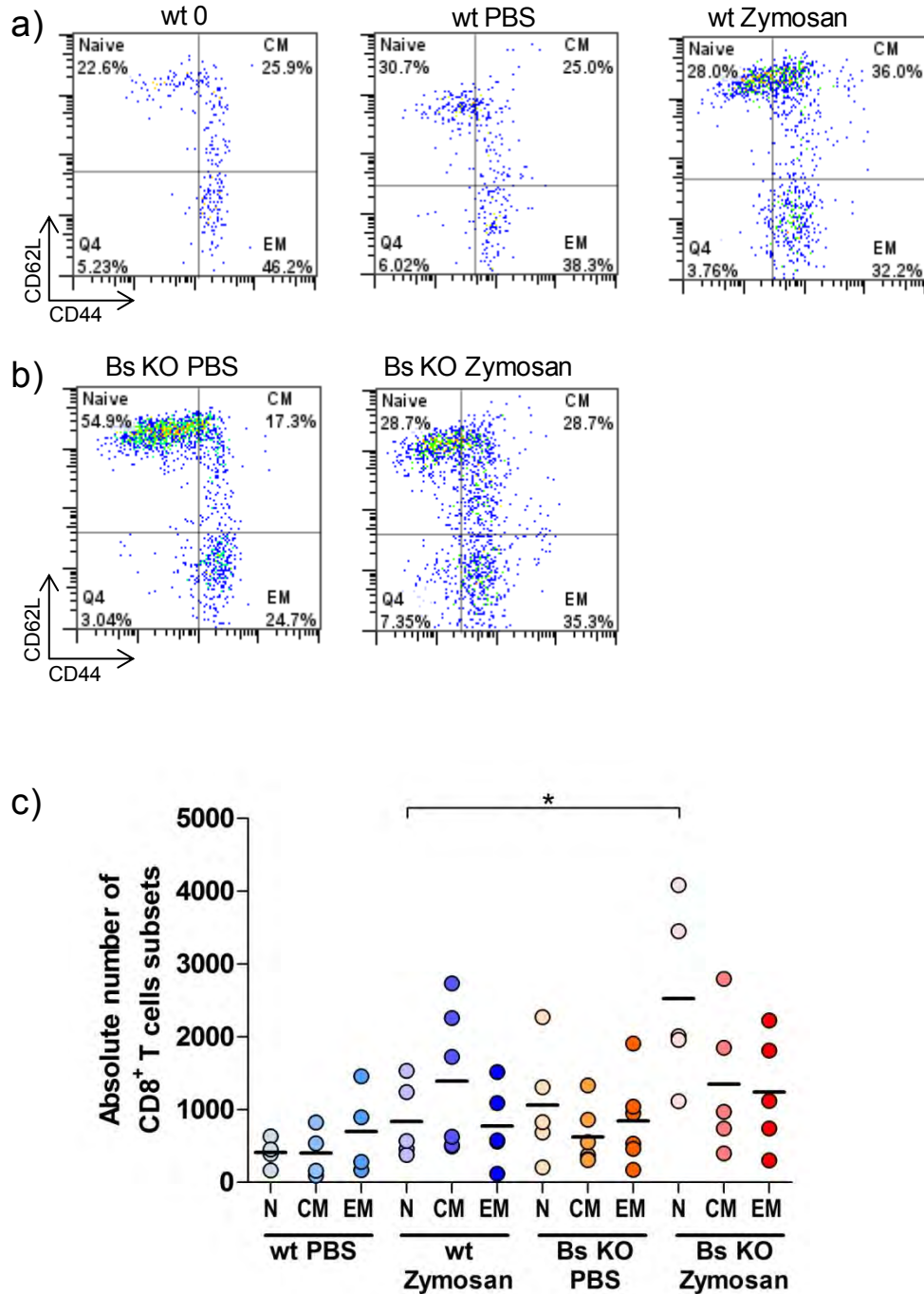


Figure 6-17: Absolute number of naive CD8⁺ T cells is slightly higher in the inflamed peritoneum of B cell knock-out mice compared to the wt mice

Leukocytes were collected from the peritoneum after 48 hours without any injection (wt 0), with PBS injection (wt PBS, Bs KO PBS) and with zymosan injection (wt zymosan, Bs KO zymosan) in (a) wt and (b) Bs KO mice. CD3⁺ and CD8⁺ T cells were first gated and naive, central memory and effector memory T cells were identified by the expression of CD44 and CD62L. Naive CD4 are CD62L⁺CD44⁻, central memory T cells are CD62L⁺CD44⁺ and effector memory T cells are CD62L⁻CD44⁺. (c) Data for each mouse groups were pooled, represented as mean and were analysed using one-way ANOVA and Bonferroni's multiple comparisons post-test. *p≤0.05.

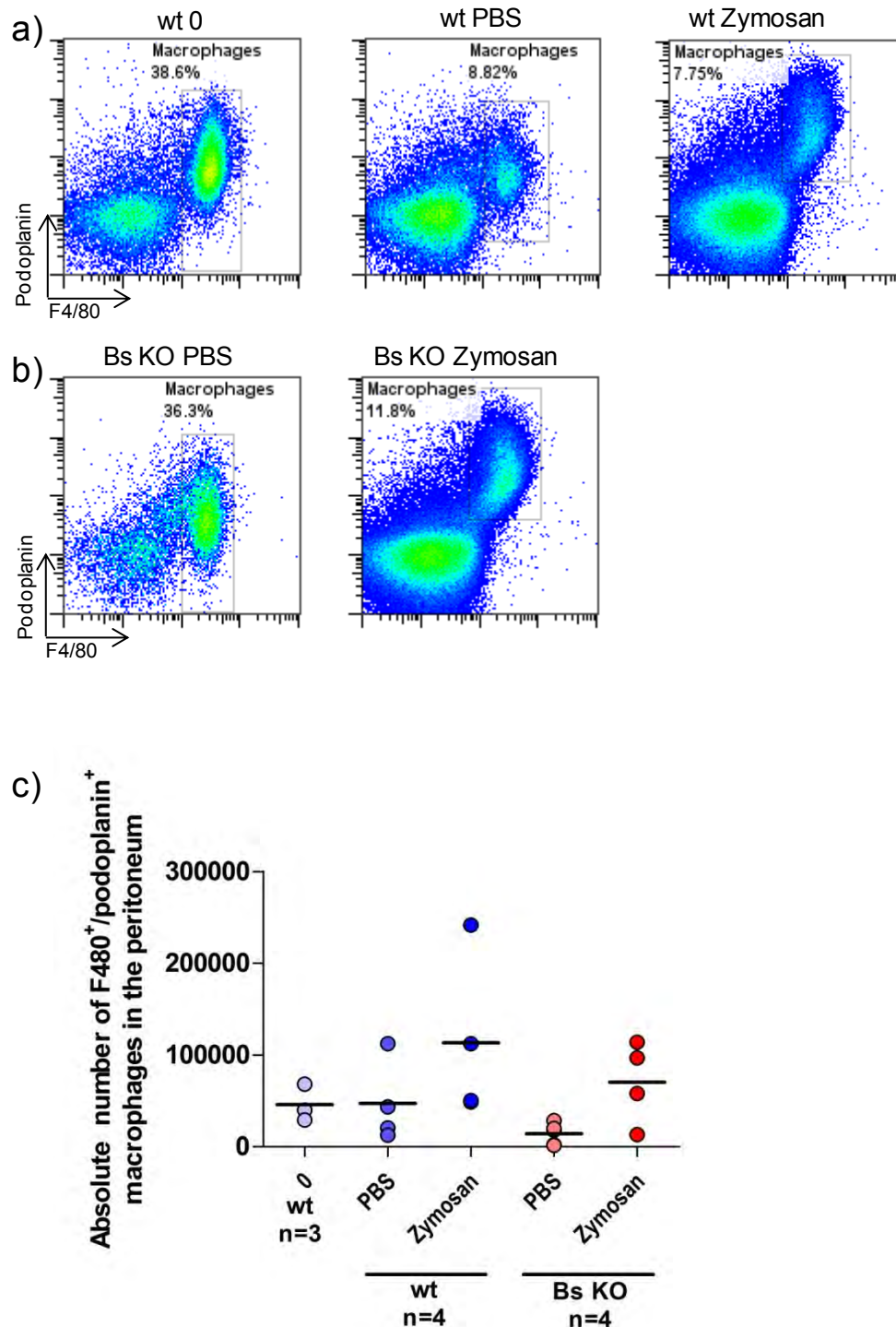


Figure 6-18: Absolute number of macrophages in the inflamed peritoneum of wt mice compared to B cell knock-out mice

Leukocytes were collected from the peritoneum after 48 hours without any injection (wt 0), with PBS injection (wt PBS, Bs KO PBS) and with zymosan injection (wt zymosan, Bs KO zymosan) in (a) wt and (b) Bs KO mice. Macrophages were identified by the expression of F4/80 and podoplanin (gp38) after gating on CD45⁺ cells and gating out CD3⁺ T cells and CD11c⁺ dendritic cells. (c) Data for each mouse groups were pooled, represented as mean and were analysed using one-way ANOVA and Bonferroni's multiple comparisons post-test. No significant differences were found.

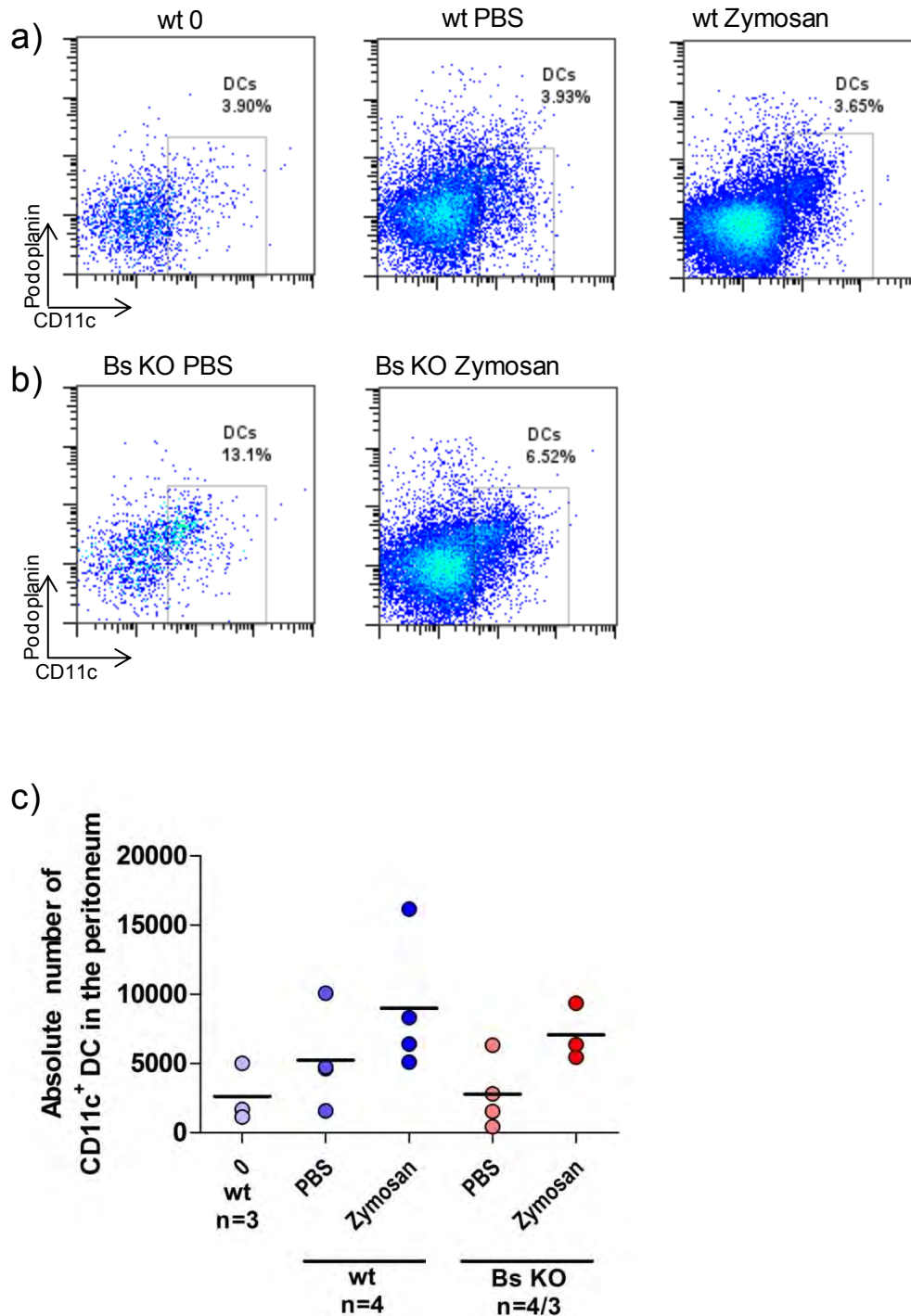


Figure 6-19: Absolute number of dendritic cells in the peritoneum is higher in inflamed conditions in both wt and B cell knock-out

Leukocytes were collected from the peritoneum after 48 hours without any injection (wt 0), with PBS injection (wt PBS, Bs KO PBS) and with zymosan injection (wt zymosan, Bs KO zymosan) in (a) wt and (b) Bs KO mice. DCs were identified by the expression of CD11c and after gating on CD45⁺ cells and gating out CD3⁺ T cells and F4/80⁺/podoplanin⁺ macrophages. (c) Data for each mouse groups were pooled, represented as mean and were analysed using one-way ANOVA and Bonferroni's multiple comparisons post-test. No significant differences were found.

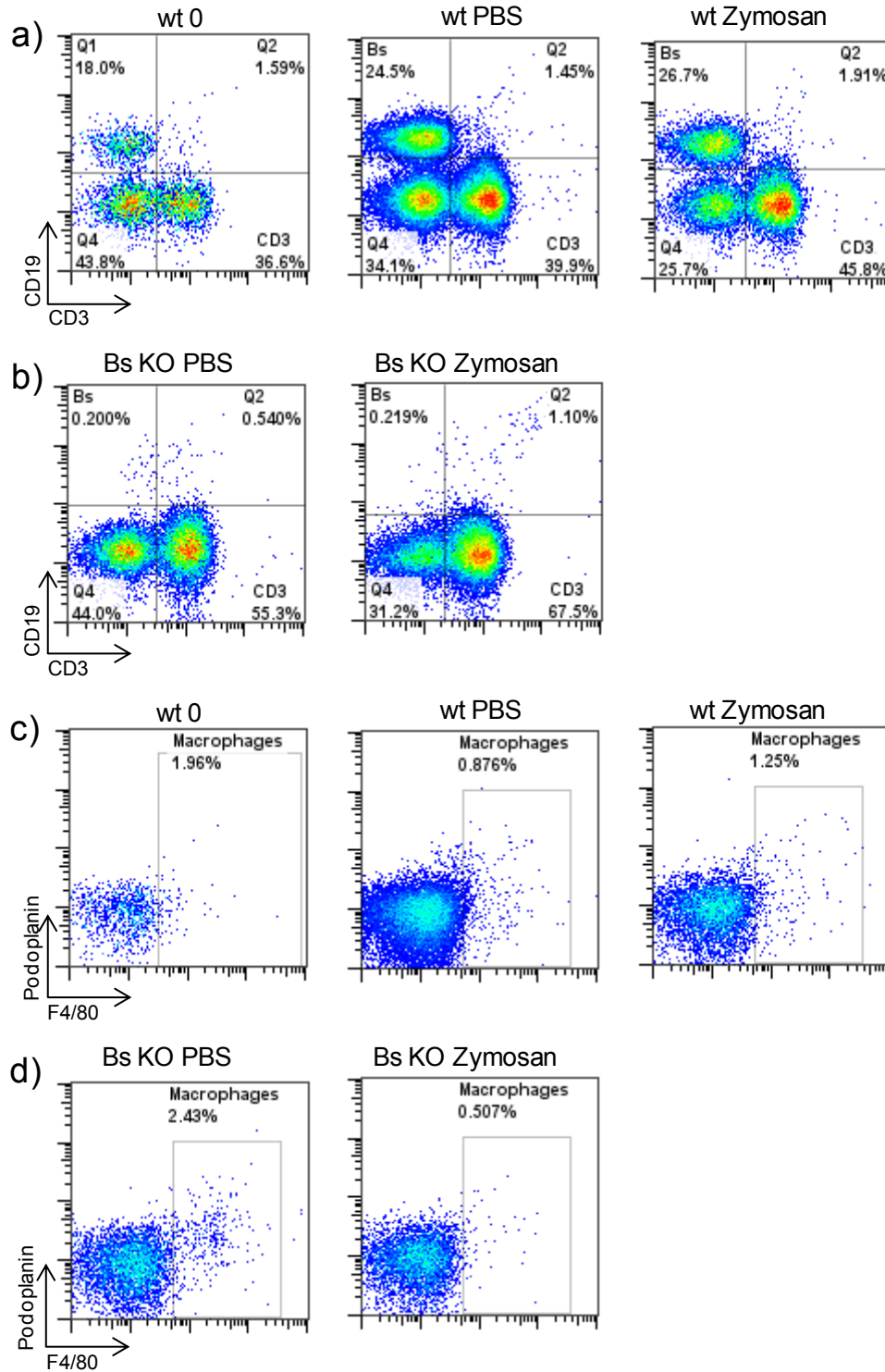


Figure 6-20: Representative dots plots of B cells, CD3+ T cells and macrophages in the blood

Blood was collected by cardiac puncture, red blood cells were lysed and leukocytes were labelled for flow cytometry. (a, b) B and T cells were identified with CD3 and CD19 in mice without any injection (wt 0), with PBS injection (wt PBS, Bs KO PBS) and with zymosan injection (wt zymosan, Bs KO zymosan) in wt and Bs KO mice. (c, d) Macrophages were identified by the expression of F4/80 and podoplanin (gp38) after gating on CD45⁺ cells and gating out CD3⁺ T cells and CD11c⁺ dendritic cells in the same mice.

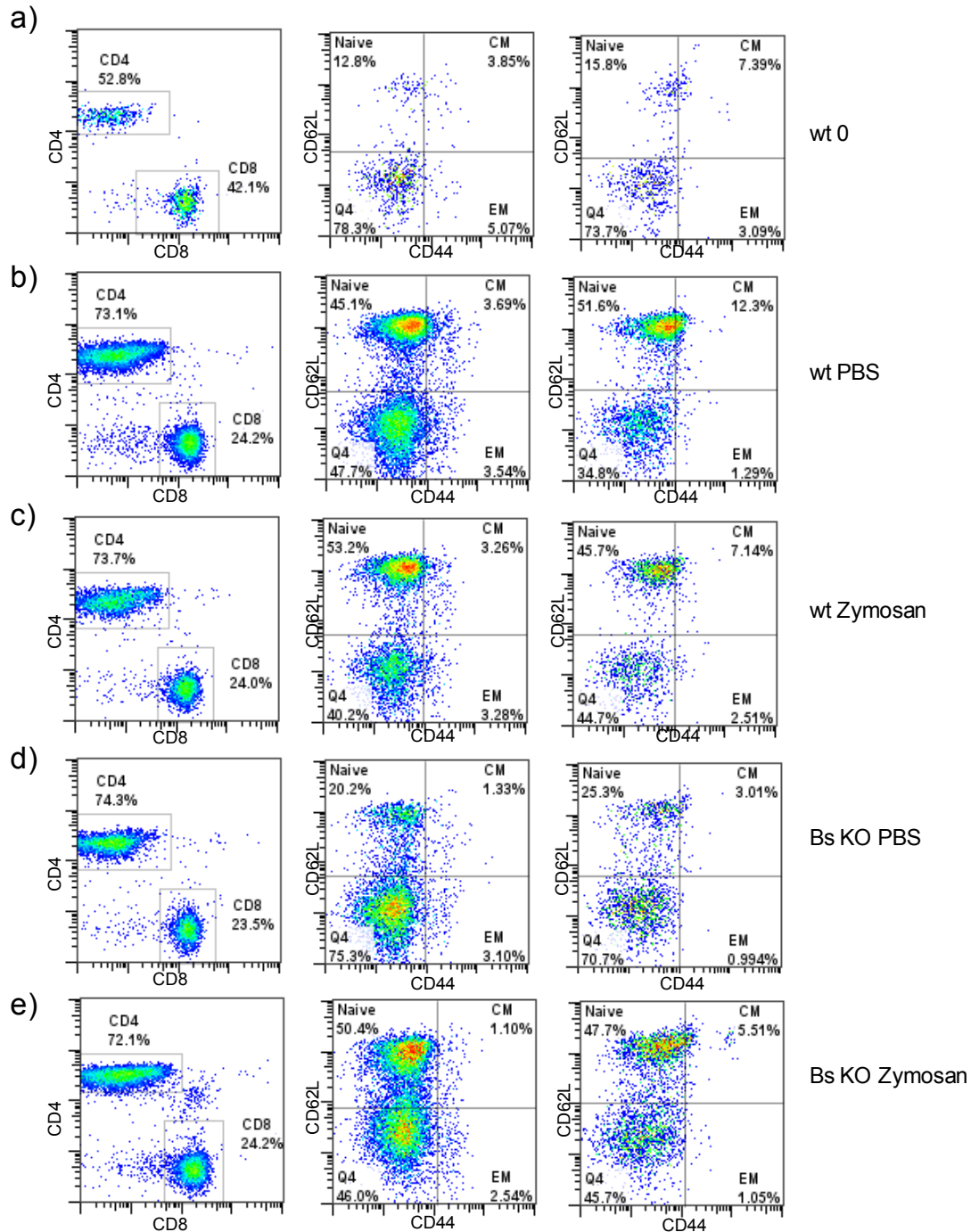


Figure 6-21: Representative dots plots of CD4 and CD8 T cells subsets in the blood

Blood was collected by cardiac puncture, red blood cells were lysed and leukocytes were labelled for flow cytometry. CD4 and CD8 T cells were gated and naive, central memory and effector memory T cells were identified by the expression of CD44 and CD62L. Naive CD4 are CD62L⁺CD44⁻, central memory T cells are CD62L⁺CD44⁺ and effector memory T cells are CD62L⁻CD44⁺ in (a) wt mice without any injection, (b) wt with PBS injection, (c) wt with zymosan injection, (d) Bs KO with PBS and (e) Bs KO with zymosan.

3. Discussion

In this chapter, we demonstrate a novel mechanism of action of adiponectin which mediates inhibition of lymphocyte trans-endothelial migration. We show that under conditions of adiponectin stimulation, B cells affect T cell transmigration. In this assay, B cells do not transmigrate and mainly plasma cells and memory B cells are recruited to the endothelium. In addition, we found that B cell adhesion is mediated via $\alpha_4\beta_1$ (VLA-4)/VCAM-1 interaction. We have confirmed these findings *in vivo* in the B cell knock-out mouse using a peritoneal model of inflammation.

- **Adiponectin is not modulating PBL transmigration in a classic manner**

A number of studies have revealed that adiponectin has pro-apoptotic effects for lymphocytes and cell lines (Pang PhD; Yokota *et al.*, 2000; Dieudonne *et al.*, 2006; Cong *et al.*, 2007; Brakenhielm *et al.*, 2004). Although, this effect is not fully understood, we sought to confirm that the inhibition of PBL transmigration we observed was not the consequence of lymphocyte apoptosis. In our system, PI staining did not reveal cell death in the presence of adiponectin and levels of the apoptotic marker, Caspase-3, were normal.

Integrins are crucial in supporting lymphocyte recruitment and modulation of their expression at the cell surface changes the capacity of lymphocytes to transmigrate through endothelium (Springer, 1994). When PBL were treated with adiponectin for one hour, there were no changes in the cell surface expression of integrins LFA-1 ($\alpha_L\beta_2$) or VLA-4 ($\alpha_4\beta_1$). Furthermore, CXCR3 signalling is essential for activation of integrins that stabilise attachment and initiate transmigration in this TNF- α /IFN- γ driven system (McGettrick *et al.*, 2009; Piali *et al.*, 1998). In addition T cell transmigration in this model also requires a PGD₂ signal downstream of chemokine stimulation to achieve efficient transmigration (Ahmed *et al.*, 2011). Indeed, in this study, PGD₂ blockade reduced T cell transmigration but not the

Chapter 6- The adiponectin-dependent inhibition of T cell migration is mediated by B cells levels of T cell adhesion to the endothelial cells. This suggests that signalling through the PGD2 receptor; DP2 is initiated after chemokine signalling and regulates migration and diapedesis rather than attachment (Ahmed *et al.*, 2011). This could be relevant to our data, as we only see changes in transmigration rather than in total adhesion. However, we found no differences in the expression of both CXCR3 and DP-2 on PBL after adiponectin treatment. In addition, we used a chemotaxis assay to check the functionality of both CXCR3 and DP-2 receptors. This assay consisted in measuring the capacity of adiponectin treated PBL (compared to untreated cells) to transmigrate through a filter towards CXCL10 or PGD2, ligands for CXCR3 and DP-2 respectively. We observed no changes in the chemotaxis of PBL towards either stimulus after treatment with adiponectin. These findings suggest that adiponectin is not modulating PBL transmigration by modulation of integrins or chemokines receptors expressions, or by changing signalling capacities of CXCR3 and DP-2.

- **Role of B cells in the mechanism of action of adiponectin**

We and others have observed an almost complete absence of the classical adiponectin receptors, AR1 and AR2, on T cells. Paradoxically however, adiponectin can regulate T cell proliferation (Wilk *et al.*, 2011). This has now been attributed to an indirect effect of adiponectin on APC such as monocytes and DCs, which do express high levels of adiponectin receptors, rather than a direct effect on T cells through an unknown receptor (Pang *et al.*, submitted). This data is compelling, as it indicates that for adiponectin to modulate T cell responses, the presence of accessory cells that express adiponectin receptor(s) is necessary. In our system, PBL subsets with such characteristic are the B lymphocytes and NK cells (Pang, Narendran, 2008; chapter 5). The former and not the later were shown to be the key regulatory cells in our new paradigm (discussed in detail below).

Interestingly, previous studies have determined that the lymphocyte recruited across unstimulated or cytokine activated endothelium are predominantly memory CD4⁺ T cells and naive and memory CD8⁺ T cells (Lichtman *et al.*, 1997; McGettrick *et al.*, 2009; Pietschmann *et al.*, 1992; Brezinschek *et al.*, 1995)). In agreement with these studies we found preferential migration of CD4⁺ and CD8⁺ memory T cells as well as CD8⁺ naive T cells in both our static and flow assays. In both systems, naive CD4⁺ T cells did not efficiently transmigrate. These subsets were also enriched on the top of the endothelium. In addition, we found that NK cells, NK T cells and CD56^{high}NK cells migrated very efficiently across the endothelium. Indeed, the transmigration of NK cells was the most efficient of any lymphocyte population. This data concurs with previous studies showing high migratory capacities of NK cells across endothelium monolayers (Allavena *et al.*, 1996). Interestingly, migration of NK cells is mediated by $\beta 2$ integrin activation upon ligation of PECAM at least for NK cells and CD56^{high}NK cells (Berman *et al.*, 1996). NK T cell adhesion and transmigration may utilise a different pathway and is reported to involve $\beta 1$ integrins (Franitza *et al.*, 2004). This study also shows that NK T cells preferentially respond to CXCR4 ligands such as CXCL12 on the endothelium.

Interestingly, B cells themselves did not transmigrate after becoming firmly adherent to the endothelium in our assay. In agreement with published data performed in flow-based assays (Yago *et al.*, 1997), we found that B cell adhesion relied on VLA-4 ($\alpha_4\beta_1$) -VCAM interaction in our static assay. One limitation of this observation is that B cells are clearly able to cross the endothelium *in vivo*, and our observations may be an artefact. For example, we have detected B cells in the lower wells of transwell experiments, where 24h was allowed for endothelial cell transmigration (unpublished observations). In addition, B cells were found in the peritoneum of wild type mice treated with zymosan, which suggest they possess an intrinsic capacity to migrate across microvascular endothelium and that lack of B cell

Chapter 6- The adiponectin-dependent inhibition of T cell migration is mediated by B cells

migration in our current assay reflects the dynamics of migration of this lymphocyte subset. Moreover, B cells migrate efficiently across HEV (Ebisuno *et al.*, 2003), although this type of endothelium is different from that in the periphery and is highly specialised for immune cell recruitment and lymphocyte trafficking (Miyasaka *et al.*, 2004). Thus, it would be worth measuring transmigration of B cells in this assay over a longer time course and to compare B and T cells kinetics. In a physiological context, it seems probable that B cells adhere to the endothelium before or at the same time as T cells. This way B cells are able to control transmigration of T cells under the influence of circulating adiponectin. We speculate that after some undefined period, B cells eventually migrate across the EC. These speculations are supported to some extent by studies in the mouse model of zymosan driven peritoneal inflammation. In these conditions, B cell numbers are low in the peritoneal exudates over the first 48 hours after zymosan administration and dramatically increase after 72 hours (Rajakariar *et al.*, 2008). In addition, studies have shown different patterns of recruitment in inflamed tissues between B and T cells. Rather than migrating and diffusing within the site of inflammation, B cells are present as clusters of tightly packed cells at restricted stromal sites (Meeusen *et al.*, 1991).

We also determined that stimulated endothelium preferentially recruited memory B cells and plasma cells form the available pool of circulating B cells. Because these subtypes are enriched at the surface of the endothelium and because the effect we observe is rapid, we believe that memory B cells and plasma cells could be the B cell subtype responsible for the effect on T cell migration, although this requires confirmation. In addition, plasma cells are highly specialised for secretion of soluble molecules so such a role would be logical. However, it was brought to our attention that plasma cells may not express CD19. Therefore, when we selectively remove B cells based on CD19 expression, we would still have the plasma cells in the assay. This would strongly mitigate against such a role for plasma cell in

Chapter 6- The adiponectin-dependent inhibition of T cell migration is mediated by B cells

our assay. However, CD19 expression by plasma cells is a subject of some controversy. Recent evidence shows circulating plasma cells are infrequently encountered in the blood. Thus, plasma cells are generated in the germinal centres of secondary lymphoid organs. Upon antigen stimulation B cells proliferate and become memory B cells or plasmablasts. Plasmablasts start secreting antibodies in the medullary cord but not as efficiently as plasma cells because they are still proliferating. The plasmablasts eventually become plasma cells, secreting large amount of antibodies, and then most plasma cells die rapidly. However a small proportion of plasma cells leave the SLOs through the circulation to enter the bone marrow where they can survive for long periods (reviewed in Shapiro-Shelef, Calame, 2005). These long-lived circulating plasma cells in blood express CD19 at low levels (Medina *et al.*, 2002; Arce *et al.*, 2004; Horst *et al.*, 2002; Harada *et al.*, 1996). In addition, they express integrins such as VLA-4 ($\alpha_4\beta_1$) which we found allows their adhesion onto EC (Medina *et al.*, 2002). They differ from plasma cells found in tonsils and their phenotype changes once they enter the bone marrow (Medina *et al.*, 2002; Arce *et al.*, 2004). In addition, the number of circulating plasma cells increases following inflammation as well as vaccination (Harada *et al.*, 1996; Shapiro-Shelef, Calame, 2005). Whether this population is substantial enough to be recruited in sufficient numbers during inflammation to regulate T cell migration is unclear, and an area requiring clarification. However, we should also acknowledge the limitations of the antibody panel we used to identify B cell subsets. This leaves some ambiguity in our attributions of subset identity and it would be worth getting a fuller panel of markers to identify all the different transitional B cells and the different memory B cells as well as plasma cells more accurately.

In conclusion, B cell depletion removes the majority of CD19⁺ B cells and we believe it also includes plasma cells. This is sufficient to show that without B cells the adiponectin-mediated inhibition of PBL transmigration is lost. However, it would be worth checking on

Chapter 6- The adiponectin-dependent inhibition of T cell migration is mediated by B cells sorted B cells subsets as well. Indeed, it is possible to sort for plasma cells and memory B cells and perform similar transmigration assays. For instance, it would be interesting to see if the effect is lost in absence of plasma cells and with reconstitution, as well as using the supernatant and Brefeldin A.

- ***In vivo* confirmation**

Here we have used a zymosan driven model of acute peritoneal inflammation in mice to define the involvement of B cells in the regulation of T cell migration *in vivo*. Zymosan is a purified polysaccharide fraction of the yeast cell wall which induces inflammation *in vivo* by activation of complement (Fearon *et al.*, 1977), induction of histamine from mast cells and basophils, induction of oxygen-free radicals and lysosomal enzymes (Bonney *et al.*, 1978), generation of free fatty acids and eicosanoids in peritoneal inflammation (Doherty *et al.*, 1987; Lundy *et al.*, 1990) and induction of pro-inflammatory cytokines by monocytes and macrophages (Sanguedolce *et al.*, 1992). Zymosan-induced inflammation is therefore a comprehensive model to study inflammation as it induces many relevant pathways.

Peritoneal inflammation has been used before to study kinetics of leukocytes recruitment during inflammation (McLoughlin *et al.*, 2005; Takada *et al.*, 1993; Rajakariar *et al.*, 2008). It can be induced by different compounds such as zymosan and or bacteria such as *E.coli* or *Staphylococcus* (Takada *et al.*, 1993; McLoughlin *et al.*, 2005). We chose zymosan induced peritonitis because of the availability of data on the kinetics of leukocyte recruitment in this model (Rajakariar *et al.*, 2008). When used at the appropriate concentration, zymosan induces a resolving acute inflammation in which neutrophils first invade the peritoneum followed by macrophages after a few hours. T lymphocytes occupying the naive peritoneum and are lost upon induction of inflammation, exiting via the draining lymphatics by action of PGD2 on the DP-1 receptor (Rajakariar *et al.*, 2008). However, after six hours, T cells start

Chapter 6- The adiponectin-dependent inhibition of T cell migration is mediated by B cells repopulating the inflamed peritoneum and numbers significantly increase above baseline levels over time. It is slightly different for B cells, as they only start to repopulate the inflamed peritoneum after 48 hours. Meanwhile, neutrophil counts decrease after 48 hours. These reports identify two phases during this inflammatory response. The first phase is characterised by innate immune-mediated inflammation and the second is more of a resolving phase involving lymphocytes. There is also a rise of NK cells, gamma/delta T cells and Treg number during the resolving phase. This has been associated with their involvement in resolution but also protection against a secondary infection. Finally, the peritoneum is restored to 'naivety' after seven days. If this is not the case, e.g. if using higher concentrations of zymosan, then the model becomes chronic and inflammation causes death.

Although this model is convenient and easy to set up, it has a few disadvantages. Analysis of the whole peritoneal exudate by flow cytometry shows a profile of cells containing a lot of debris and red blood cells. Thus, to be able to compare results between mice and determine the absolute number of cells, it was important to gate on the whole leukocyte populations in a similar manner for each mouse. We excluded red blood cells and debris based on the forward/side scatter profile and then excluded duplets of cells, which allowed us to get a cleaner profile. After optimisation of the staining, we managed to easily identify CD3⁺ T cells and their subsets, as well as DCs and macrophages.

In addition, this is a cavity based model and therefore does not involve tissue specific responses. Another way to look at inflammation in tissues would be to use the canulation technique. Using this method, inflammation could be induced in salivary glands for instance, and this would allow us to look at specific recruitment into tissues in the B cell KO model. Although, zymosan is well characterised as an inducer of inflammation in the peritoneum, other stimuli have been used and may be worth consideration. In summary, although this model provides good evidence for a role of B cells during T cell recruitment in an

inflammatory response, it would be interesting to analyse this in other models. In fact, we have utilised B cell knock-out mice on the C57BL/6 background in a model of *Salmonella typhimurium* induced hepatic inflammation (in collaboration with Dr Adam Cunningham, University of Birmingham). We found that the number of T cells per infection loci in the liver was higher in B cell-deficient mice compared to wt (data not shown). Although, this is a model of infection, inflammation is also a part of the infectious processes. These data consolidate our findings in another model of inflammation but also in another mouse strain.

Finally, we did not investigate adiponectin levels in the mice included in the study. Serum samples were collected and it would be worth measuring adiponectin levels in these mice. It has been shown that circulating adiponectin levels decrease during zymosan-induced inflammation but no changes was observed in the peritoneal exudates (Pini *et al.*, 2008). It would be interesting to confirm this in our mice.

- **Conclusions**

To summarize, we provide evidence for a novel role for B cells in regulating T cell recruitment in the presence of adiponectin. These effects were validated *in vivo*, using a model of inflammation in the B cell knock-out mice.

7.Chapter 7- A NOVEL ADIPONECTIN- INDUCED, B CELL-DERIVED PEPTIDE, PEPITEM, INHIBITS THE MIGRATION OF T CELLS

1. Introduction

We clearly show that B cells modulate T cell migration *in vitro* and *in vivo*. In this chapter, we aimed to understand how B cells modulate T cell migration. We therefore investigated whether the B cells act on the T cells by secreting an agent or by cell to cell contact. We then aimed to identify potential soluble candidates secreted by B cells and we tested their biological activity in our model of PBL transmigration.

2. Results

2.1. B cells modulate T cell migration through a secreted agent X

Here, to test whether B cells secrete an agent that modulates T cell transmigration, B cells were isolated as previously described and incubated with adiponectin for one hour. The supernatant of B cells was then added to the Bs-ve PBL for 30 minutes and transmigration was measured as previously described. Additionally, B cells were treated with Brefeldin A (BrefA), an inhibitor of protein transport from the endoplasmic reticulum to the Golgi which essentially inhibits secretion in B cells. After three hours incubation with BrefA, adiponectin was added to the B cells for one hour and the BrefA B cells were used to reconstitute the Bs-ve PBL (Bref A Bs PBL). As previously described, we found that B cell depletion resulted in loss of the inhibition of transmigration mediated by adiponectin (**Figure 7-1**). Adding the supernatant of B cells treated with adiponectin to the Bs-ve PBL restored the inhibition of transmigration. Importantly, the blockade of protein secretion with BrefA ablated the B cell-mediated inhibition of PBL transmigration. These results indicate that inhibition of PBL trans-endothelial migration by adiponectin is mediated by an agent secreted by B cells.

However, supernatant of B cells added to the Bs-ve PBL fraction, contains adiponectin which cannot be removed from the supernatant before treatment. As indicated earlier,

HUVEC express the adiponectin receptors (Chapter 5) and treatment of HUVEC with adiponectin also reduces PBL transmigration. To check that adiponectin is not directly acting on the HUVEC in the B cell supernatant experiment, we went on to knock down both adiponectin receptors on HUVEC. We achieved an average of 90% knock-down for AR1 and 80% for AR2 (**Figure 7-2a**). Under these conditions, the supernatant of B cells treated with adiponectin still inhibited PBL transmigration (**Figure 7-2b**). These data therefore confirm that in experiments using B cell supernatants, it is a secreted agent and not adiponectin which inhibits T cell transmigration.

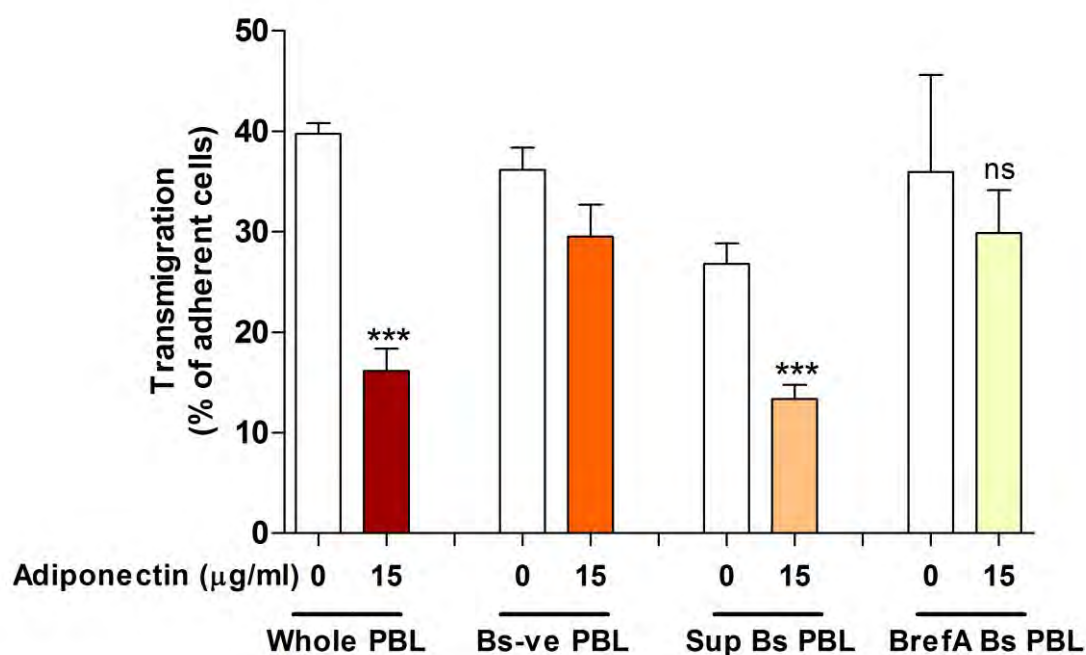


Figure 7-1: B cells modulate PBL transmigration through a secreted agent X

B cells were isolated and incubated in presence or absence of adiponectin at 15µg/ml. Supernatant was taken after one hour and added to Bs-ve PBL which significantly restored the adiponectin inhibition of PBL transmigration. For some conditions, B cells were treated with Brefeldin A for four hours and adiponectin after 3 hours and added back to Bs-ve PBL. This induced loss of the adiponectin effect. Data are shown as mean±SEM, are a pool of three independent experiments and analysed using one-way ANOVA and Bonferroni's multiple comparison post test. ***p<0.001, ns=non significant.

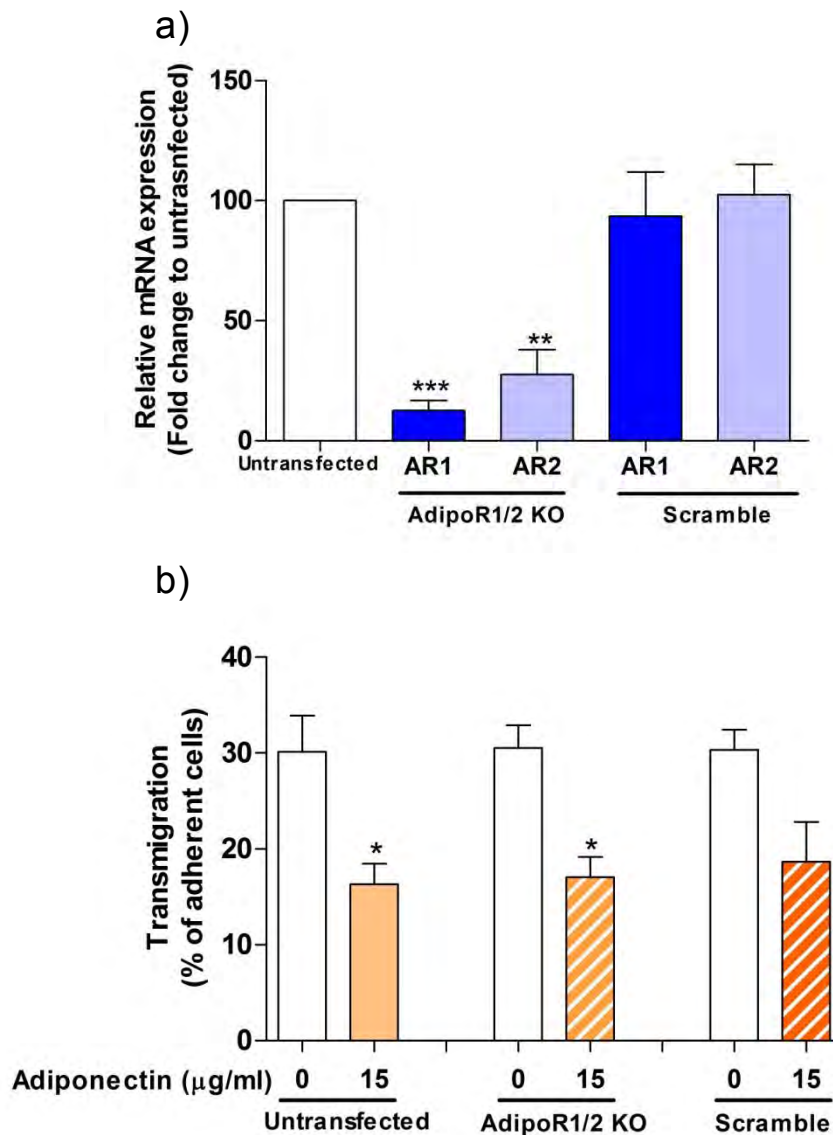


Figure 7-2: Knock-down of adiponectin receptors on HUVEC does not affect inhibition of transmigration by the secreted Agent X

(a) HUVEC were transfected with siRNA against AR1 and AR2 or scrambled versions of the target siRNA. (b) After 48 hours, HUVEC were stimulated with $\text{TNF-}\alpha/\text{IFN-}\gamma$ and PBL transmigration was measured on Bs-ve PBL reconstituted with B cell supernatant with or without adiponectin. In these conditions, inhibition of PBL transmigration by Agent X contained in B cell supernatant was not affected by the absence of AR1/2 on the endothelium. Data are shown as mean \pm SEM, are a pool of three experiments and were analysed using one-way ANOVA and Bonferroni's multiple comparison post test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

2.2. Adiponectin-mediated inhibition of PBL trans-endothelial migration is mediated by SIP

Next, we aimed to identify the B cell-secreted Agent X. Our first candidate was sphingosine-1-phosphate (S1P), because of its well-described role in the control of T cell migration. Studies have shown that S1P binds to two GPCR receptors on T cells, S1P receptor 1 and 4 (S1P1, S1P4) in mice and humans (Matloubian *et al.*, 2004; Graeler, Goetzl, 2002). These receptors have been detected only at an RNA level or by western blot in human T cells, but never using flow cytometry. We purchased rabbit primary antibodies against the receptors and optimised the staining of both S1P1 and 4, which allowed us to quantify each receptor, either on the surface or intracellularly, on T cells using flow cytometry. We found that both S1P1 and 4 were minimally expressed at the cell surface (**Figure 7-3a**). Interestingly however, the frequency of S1P1 positive cells, but not S1P4 positive cells was significantly increased on PBL that have transmigrated (**Figure 7-3b, c**). Further analysis revealed that both receptors are highly expressed intracellularly (**Figure 7-4a**). We observed no significant differences in the frequency of positively staining cells, or the MFI when comparing whole PBL, firmly adhered cells, and migrated cells. However, significantly higher MFI were found for intracellular S1P4 in all conditions analysed (**Figure 7-4b, c**).

In summary, whole PBL express both S1P1 and S1P4 at the surface at low levels and at high levels intracellularly.

In order to show the involvement of S1P in the adiponectin effect on PBL transmigration, we aimed to block the S1P receptors using a specific antagonist. Thus, whole PBL or Bs-ve PBL were treated for 30 minutes with the S1P1/4 antagonist, W146. Cells were then washed and PBL were treated with adiponectin for an additional hour and subjected to transmigration on TNF- α /IFN- γ stimulated HUVEC. Bs-ve PBL were reconstituted with B cells treated with adiponectin for one hour. For both conditions, pre-treatment with the S1P

receptor antagonist reversed the adiponectin-mediated inhibition of PBL transmigration (**Figure 7-5**). This suggests that the adiponectin effect is mediated via the S1P receptors on T cells.

We went on to investigate the direct action of S1P on transmigration in Bs-ve PBL preparations. As these B cell depleted PBL lack a response to adiponectin, we might expect a restoration of inhibition of PBL transmigration by S1P, if S1P is the B cell-secreted agent. Bs-ve PBL were treated with S1P at 0.0001 to 100 μ M for 30 minutes. Interestingly, S1P induced a significant dose-dependent inhibition of transmigration, which was as potent as the adiponectin effect and at high concentrations (**Figure 7-6a**).

In parallel, we looked at the time course of inhibition of migration in these experiments. This analysis revealed that S1P-induced inhibition of PBL transmigration was lost over time, as was the effect of adiponectin (**Figure 7-6b, c**), interestingly with very similar kinetics. In addition, we ensured that PBL treated with S1P were alive. This was confirmed by the absence of PI staining after S1P treatment (**Figure 7-7**). In conclusion, as we reveal a loss of the effects of adiponectin after antagonising S1P receptors, and as exogenous S1P can reconstitute the effects of adiponectin treatment, it is possible that this molecule is the agent secreted by B cells in response to adiponectin.

However, production of S1P by B cells requires expression of the sphingosine kinases 1 and/or 2 (SPHK1, SPHK2). Indeed, these enzymes are essential for the phosphorylation of sphingosine leading to S1P production. Thus, we analysed the mRNA expression of both enzymes in B cells and HUVEC. Surprisingly, B cells did not express SPHK1 and SPHK2. However, SPHKs, especially SPHK1, were expressed at high levels in HUVEC (**Figure 7-8a**). Interestingly, stimulation of HUVEC with TNF- α /IFN- γ induced a slight but not significant up-regulation of SPHK1 mRNA expression (**Figure 7-8b**). Therefore we had to modify our model. It now appears that S1P was not the B cell-secreted agent, although S1P,

Chapter 7- A novel adiponectin-induced, B cell-derived peptide, PEPITEM, inhibits the migration of T cells probably derived from endothelial cells was responsible for inhibiting T cell migration. Thus, it would appear that S1P is released from the endothelium upon stimulation by another agent released from adiponectin-stimulated B cells

To verify this paradigm, we aimed to confirm that the production of S1P by HUVEC was responsible for the adiponectin-mediated inhibition of PBL transmigration. Thus, we depleted HUVEC for SPHK1/2 using siRNA duplexes. However, we could only obtain low knock-down efficiency for SPHK1 ($\approx 40\%$) (**Figure 7-9a**). Knock-down of SPHK2 was more efficient ($\approx 82\%$) Even a double transfection protocol for SPHK1 did not deliver efficient knock-down. Consequently, adiponectin-mediated inhibition of PBL transmigration was not reversed on HUVEC with SPHK1 and/or SPHK2 knock-down (**Figure 7-9b**). Due to the poor efficiency of knock-down it is not possible to draw firm conclusions from these experiments.

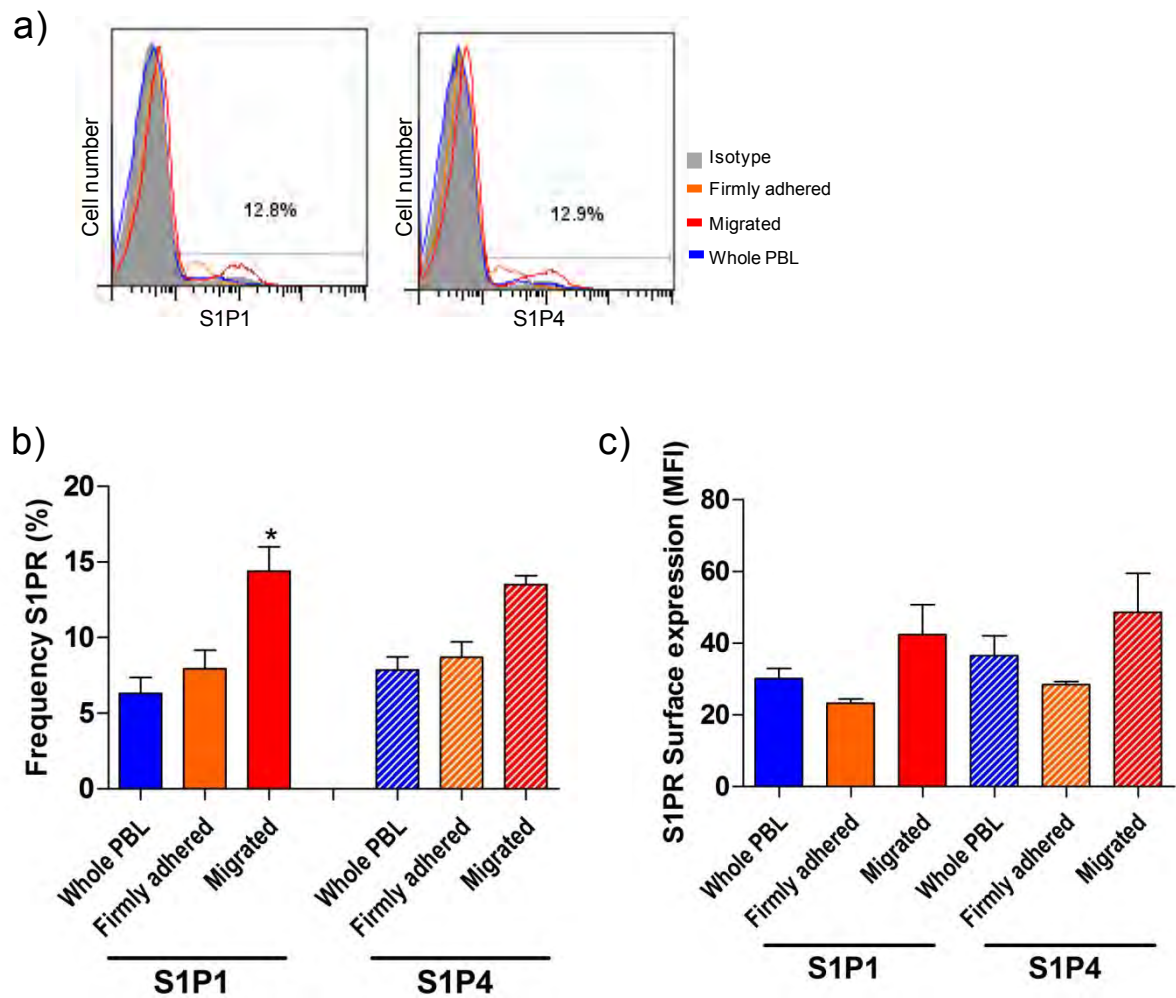


Figure 7-3: Surface expression of S1P1 and 4 on whole PBL

(a) S1P1 and 4 surface expression was measured by flow cytometry on whole PBL (blue), firmly adhered PBL to the endothelium (orange) and transmigrated PBL (red). Histograms for both receptors show little surface expression. (b) The frequency of PBL expressing S1P1 is significantly increased on transmigrated PBL but no significant differences were found for S1P4 and for (c) the surface expression of both receptors expressed as MFI. However, there was generally a trend to an increase of S1P1 and 4 expressions on transmigrated PBL. Data are shown as mean \pm SEM, are a pool of three donors and were analysed using one-way ANOVA and Bonferroni's multiple comparison post test. * $p \leq 0.05$.

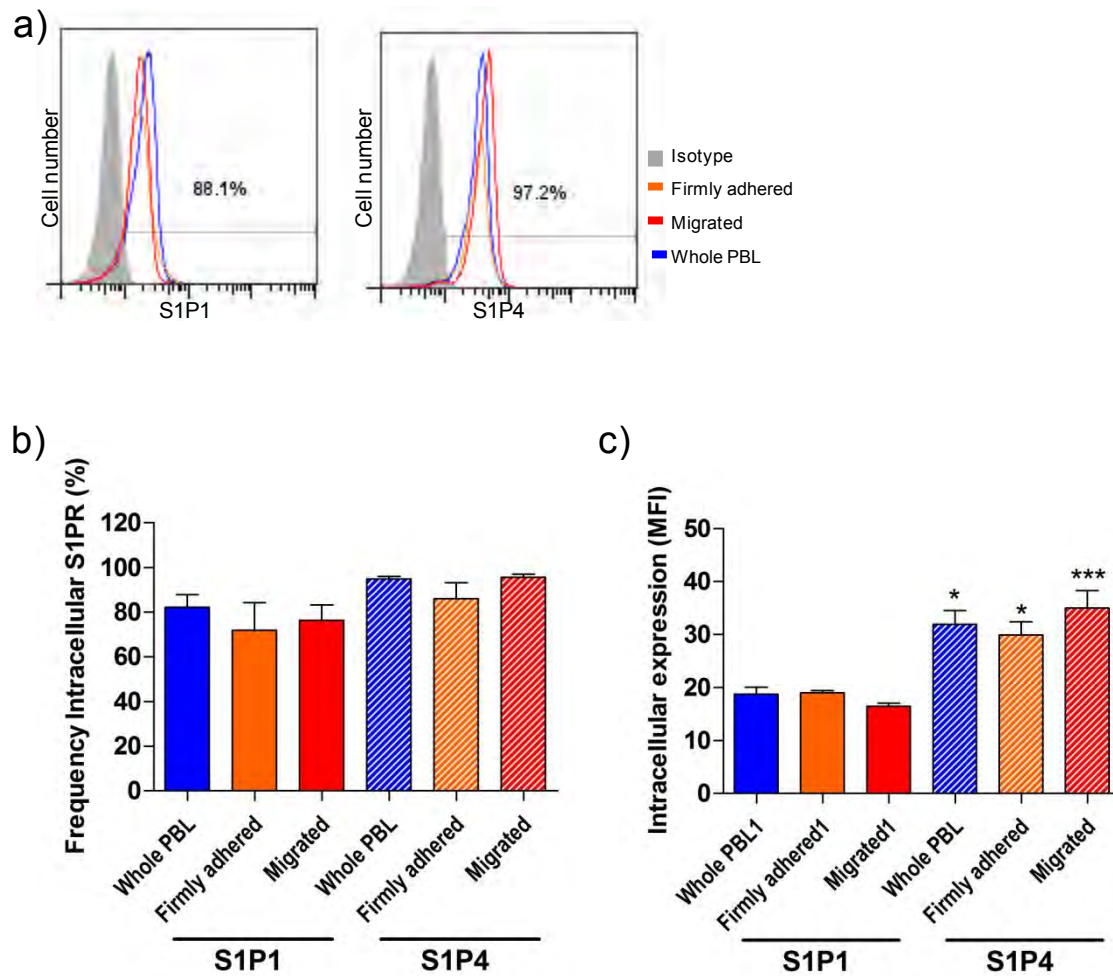


Figure 7-4: Intracellular expression of S1P1 and 4 on whole PBL

(a) S1P1 and 4 intracellular expression was measured by flow cytometry on whole PBL (blue), firmly adhered PBL to the endothelium (orange) and transmigrated PBL (red). Histograms for both receptors show high intracellular expression. (b) The frequency of PBL expressing S1P1 and 4 and (c) the expression (MFI) were not significantly different between whole PBL, firmly adhered or migrated conditions. However, significantly higher MFI of S1P4 was found. Data are shown as mean \pm SEM, are a pool of three donors and were analysed using one-way ANOVA and Bonferroni's multiple comparison post test. * $p\leq 0.05$, ** $p\leq 0.01$, *** $p\leq 0.001$.

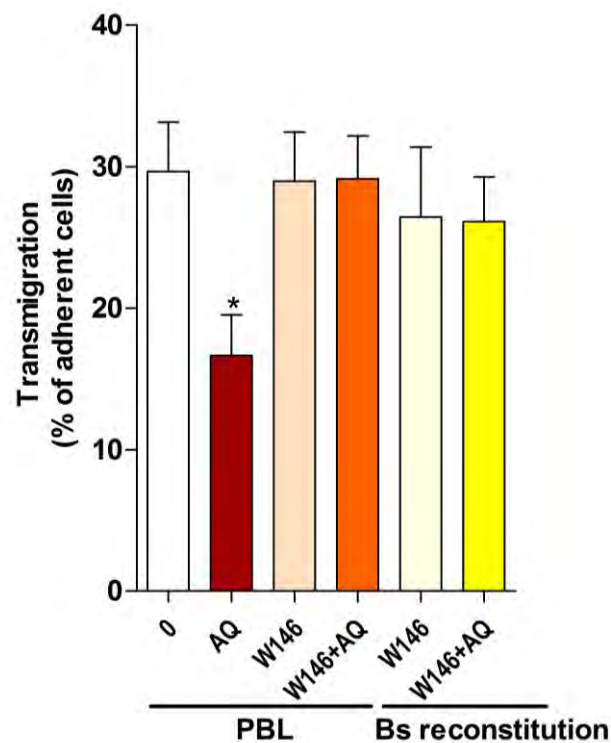


Figure 7-5: Blockade of S1P receptor abrogates adiponectin-mediated inhibition of PBL transmigration

PBL or B cells were pre-incubated with S1PR antagonist W146 at 1 μ M for 40 minutes, followed by one hour treatment with adiponectin (AQ) in some conditions. Bs-ve PBL were then reconstituted as described before with the antagonist+adiponectin treated B cells. Transmigration was measured by phase contrast videomicroscopy on TNF- α /IFN- γ stimulated HUVEC. Data are shown as mean \pm SEM, are a pool of three independent experiments and were analysed using one-way ANOVA and Bonferroni's multiple comparison post test. * $p \leq 0.05$.

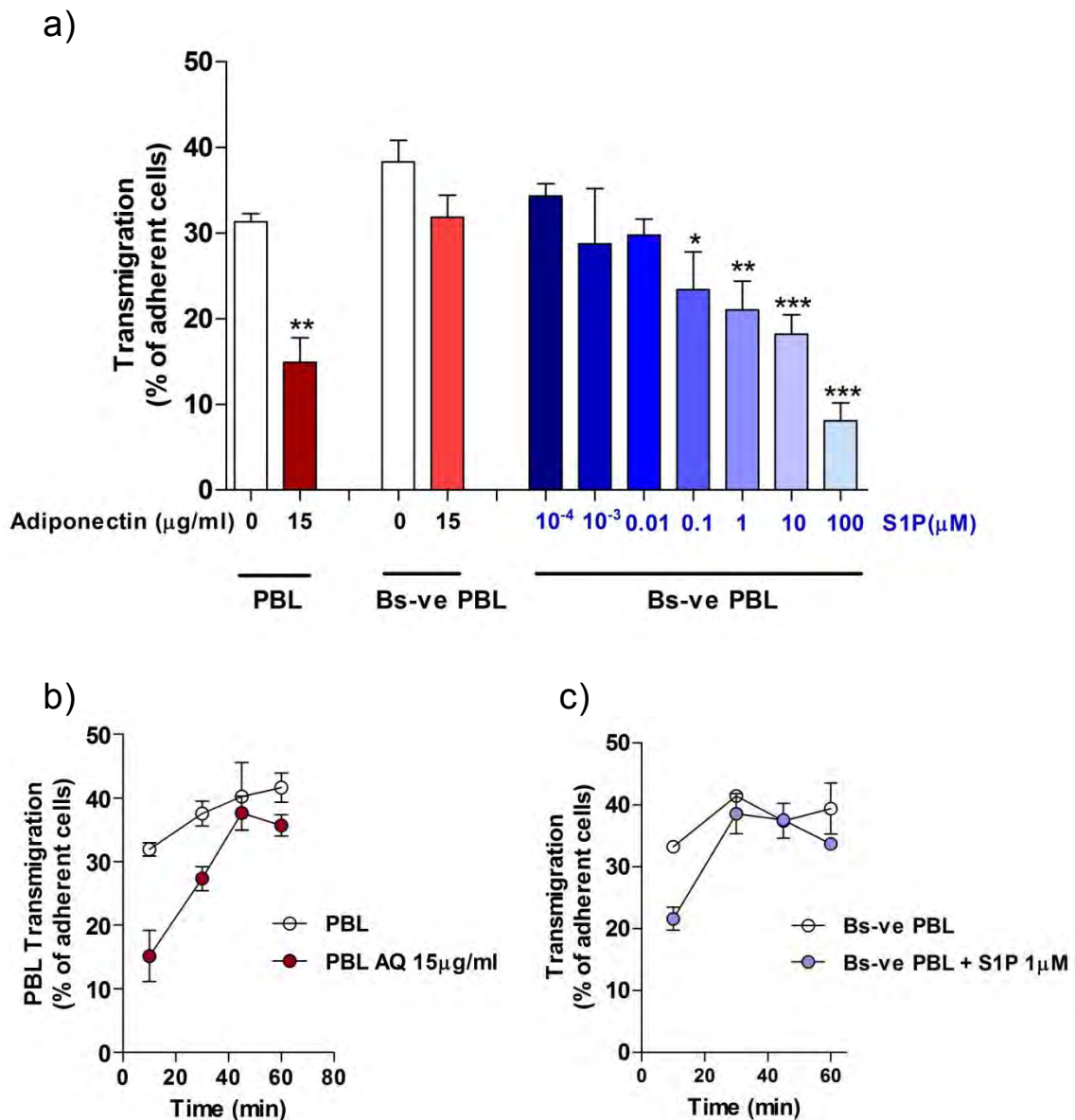


Figure 7-6: S1P inhibits transmigration of PBL depleted for B cells

(a) PBL were depleted for B cells (Bs-ve PBL) and treated with S1P at 0.0001 to 100 μM for 30 minutes. Transmigration on TNF- α /IFN- γ stimulated HUVEC was measured using phase contrast videomicroscopy. (b) PBL were treated with adiponectin (AQ) for one hour as previously described. Transmigration of PBL with or without adiponectin and (c) of Bs-ve PBL with or without S1P was measured over time starting at the usual 8-10 minutes time point and every 15 minutes until one hour. In these conditions S1P induced inhibition of transmigration which was lost over time as drastically as the adiponectin mediated inhibition. Data are shown as mean \pm SEM, are pool of at least three independent experiments and were analysed using one-way ANOVA and Bonferroni's multiple comparison post test. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

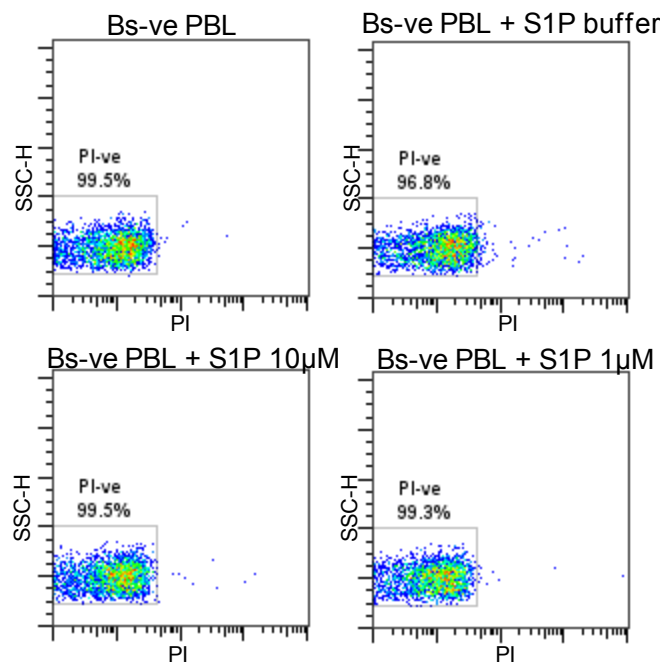


Figure 7-7: S1P treatment is not cytotoxic

PBL depleted for B cells (Bs-ve PBL) were treated with S1P at 1 and 10µM and the buffer used to resuspend the stock solution of S1P. After washes, cells were stained with propidium iodide (PI) a marker of dead cells and analysed by flow cytometry. No differences were observed in presence or absence of S1P. Data are representative of two independent experiments.

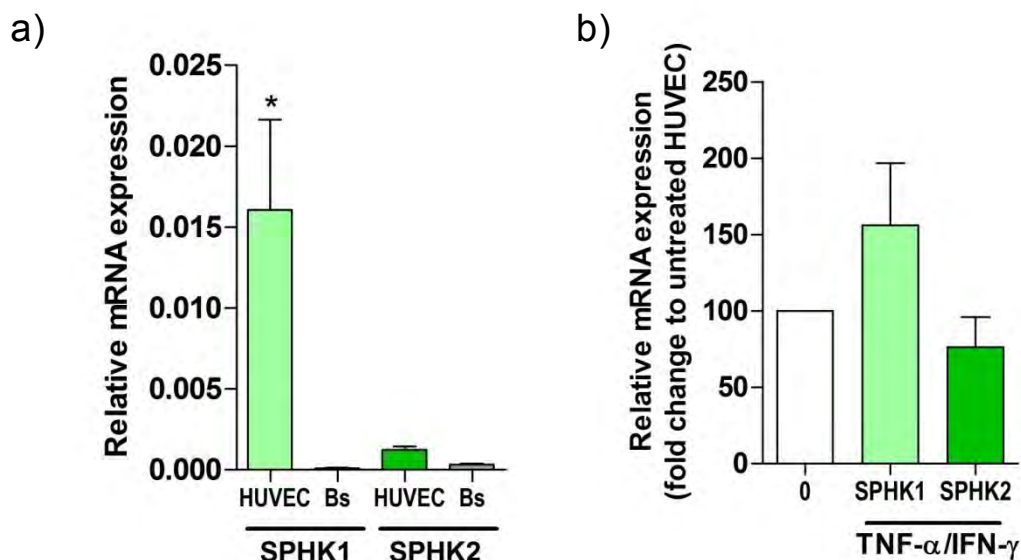


Figure 7-8: HUVEC express high levels of SPHK1

mRNA of unstimulated and TNF-α/IFN-γ stimulated HUVEC and B cells was collected and converted into cDNA. (a) Real-time PCR for sphingosine kinase 1 and 2 (SPHK1 and 2) were analysed as relative expression of SPHK1 and 2 to the endogenous control. (b) Fold change to the unstimulated control HUVEC was also calculated compared to stimulated conditions. Data are shown as mean±SEM, are a pool of at least 2 independent experiments and were analysed using one-way ANOVA and Bonferroni's multiple comparison post test. *p≤0.05.

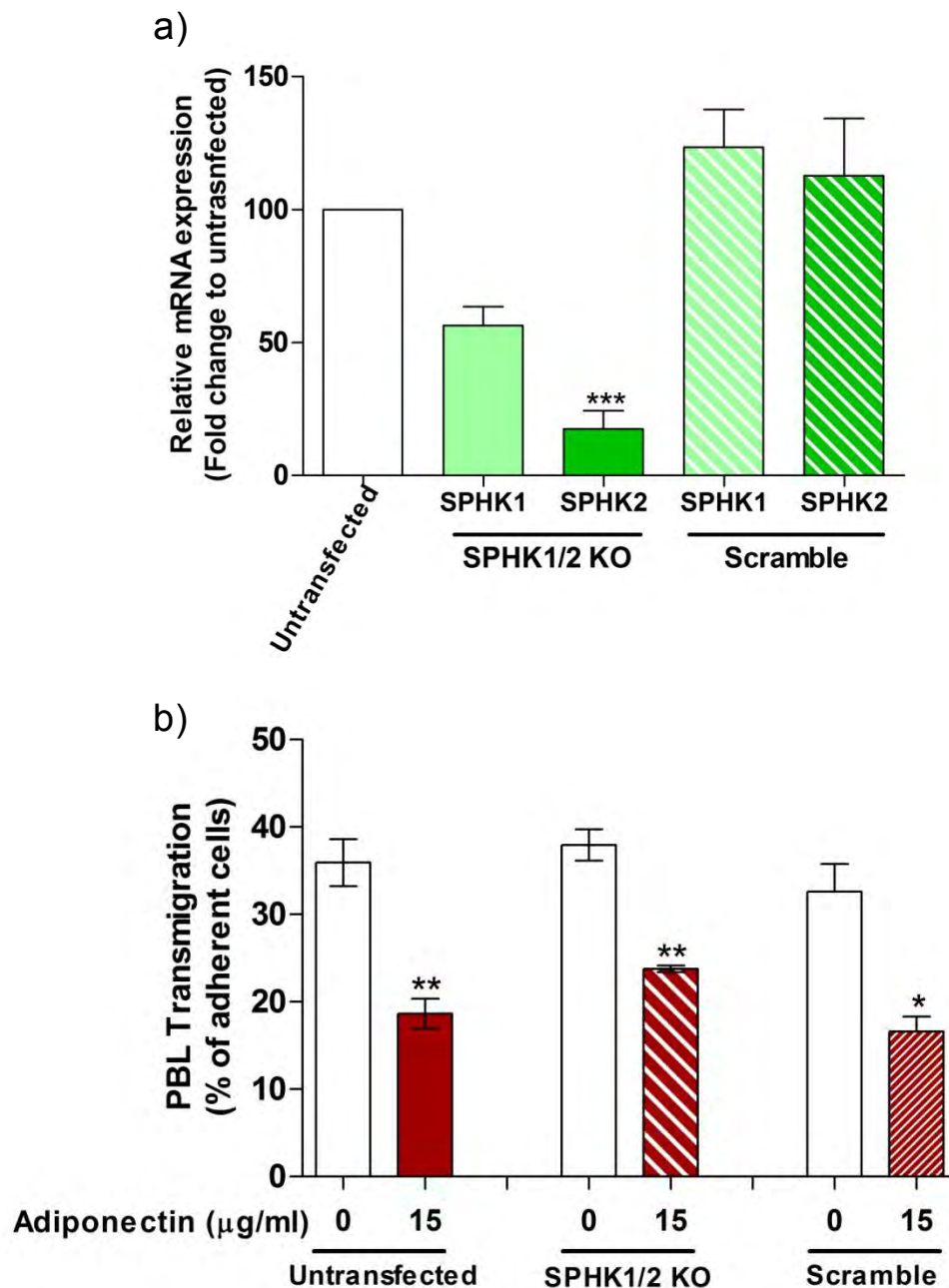


Figure 7-9: Effect of SPHK1/2 knock-down on PBL transmigration

(a) HUVEC were transfected with siRNA against SPHK1 and SPHK2 or scramble versions of the target siRNA. After 24 hours, HUVEC were re-transfected with the relevant siRNA pool and were stimulated with TNF- α /IFN- γ . PBL transmigration was measured by phase-contrast videomicroscopy in presence or absence of adiponectin. SPHK1 knock-down was not as effective as SPHK2. (b) Therefore PBL transmigration in SPHK1/2 knock-down HUVEC in presence of adiponectin was unchanged compared to the untransfected control. Data are shown as mean \pm SEM, are a pool of three independent experiments and were analysed using one-way ANOVA and Bonferroni's multiple comparison post test. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

2.3. Identification of the secreted Agent X by mass spectrometry

We established that S1P is not the B cell-secreted agent. Instead, under adiponectin stimulation B cell seem to be able to produce an unidentified agent that triggers S1P production by HUVEC, and that in turn inhibits PBL transmigration. In collaboration with Dr Ashley Martin, we used a proteomics approach to identify this agent.

Proteomics is the large scale study of proteins and peptides contained in a complex biological mixture. It allows identification of proteins, determination of their structure and the presence of post-translational modifications. Protein mixtures are analysed using mass spectrometry analysis (principle of the method used are described in Chapter 2-section 2.8.2). Identification of proteins in a biological sample is usually achieved by fragmentation of the proteins into peptides using trypsin. The specific profile of fragmentation acquired upon mass spectrometry is analysed by a software attached to a sequence database which allows identification of the consequent proteins based on their fragmentation profiles. Identification of peptides into a biological sample does not require use of trypsin and this method therefore represented a logical first approach to identify the B cell-derived agent.

B cells were incubated with or without adiponectin for one hour and the supernatants were collected. The supernatants as well as similar volume and concentration of recombinant adiponectin were then purified on a C18 column to remove potential contaminants and large size proteins. Samples were analysed by LC-MS/MS using a gradient of 2 to 36% acetonitrile on the HPLC column. Data were analysed using the Mascot search engine for the SwissProt protein database. This generated the three lists: **Table 7-1** for B cell supernatant with adiponectin, **Table 7-2** for B cells supernatant without adiponectin and **Table 7-3** for the recombinant adiponectin control. These lists archive the peptides originating from these samples and associate them with parent proteins. Mascot performs mass spectrometry analysis through a statistical evaluation of matches between observed and projected fragments from

primary sequences databases. It therefore calculates a score that indicates the quality of the match between the mass of the peptide from the mass spectrometry analysis and the real mass in the database, the higher the score, the closer match. Scores above 30 for individual peptides represent stringent statistical matches (as advised by the software manufacturer). However, in these tables the scores are the sum of each peptide identified for a given protein. The list of proteins is organised in descending order of score, and because of space constraints, we only provided the first 10 hits with the highest scores. To identify a potential candidate in the B cell supernatant with adiponectin treatment, we also applied a subtractive data analysis method. Hits from the adiponectin stimulated sample were compared to the B cell supernatant without adiponectin and recombinant adiponectin controls and common analytes were discarded. Proteins with high peptide scores after subtractive analysis are listed in **Table 7-4**. Only one high scoring peptide was identified with this method (**highlighted in red in Table 7-1**).

The 14 amino acid peptide belongs to the 14-3-3 zeta/delta protein with a matching score of 63.1. It was eluted at 13.2 minutes and was absent from the base peak chromatogram (BPC) of the B cell supernatant without adiponectin (**Figure 7-10**). The parent ion for the identified peptide has an m/z (mass/charge ratio) of 774.88 (**Figure 7-11**). This m/z parent ion peak is absent from the B cell supernatant without adiponectin, confirming specificity (**Figure 7-12**). MS/MS of the m/z 774.88 parent ion was only possible by collision induced dissociation (CID) and not by electron transfer dissociation (ETD) (**Figure 7-13**). The MS/MS gave a specific profile of fragmentation that allowed identification of the peptide sequence: SVTEQGAELSNEER (**Figure 7-14**). We acquired a synthesised version of the peptide, referred to as standard. BPC analysis revealed that the synthetic reagent was very pure and importantly, found the same m/z of 774.88 for the parent ion (**Figure 7-15**). The elution time was slightly different as the compound was eluted at 15.7 minutes but this was

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migration of T cells

explained by slight changes in the acetonitrile elution gradient used in this particular experiment. Finally, comparison of the MS/MS for the identified peptide and the standard revealed an almost complete match between the two fragmentation profiles (**Figure 7-16**).

Taken together, these data allowed identification of a 14 amino acid peptide as the candidate agent released from adiponectin stimulated B cells. This peptide was not found in the relevant controls. The peptide sequence was confirmed using the synthesised standard that had an identical m/z, similar time of elution and matching MS/MS fragmentation profile. The peptide will now be referred as PEPITEM.

Hit Nb	Accession	Protein	MW [kDa]	Similar Proteins	Scores	Peptides	RMS90 [ppm]
1	TITIN_HUMAN	Titin	3813.8	5	128.4 (M:128.4)	7	580.66
2	LRP1_HUMAN	Prolow-density lipoprotein receptor-related protein 1	504.2	1	116.9 (M:116.9)	6	169.37
3	MUC6_HUMAN	Mucin-6	252.0	1	96.0 (M:96.0)	5	1087.98
4	LAMA2_HUMAN	Laminin subunit alpha-2	343.7	1	65.0 (M:65.0)	3	171.98
5	1433Z_HUMAN	14-3-3 protein zeta/delta	27.7	1	63.1 (M:63.1)	1	25.23
6	CO1A2_HUMAN	Collagen alpha-2(I) chain	129.2	1	61.5 (M:61.5)	2	993.21
7	KDM6B_HUMAN	Lysine-specific demethylase 6B	180.3	1	60.1 (M:60.1)	2	533.71
8	WNK2_HUMAN	Serine/threonine-protein kinase WNK2	242.5	1	59.9 (M:59.9)	2	121.81
9	PGBM_HUMAN	Basement membrane-specific heparan sulfate proteoglycan core protein	468.5	6	55.4 (M:55.4)	3	339.06

Table 7-1: Results of the Mascot database search on B cell supernatant with adiponectin

Data representative of the nine highest score detected after Mascot human protein database search which resulted in 264 hits. Protein hits are classed by descendant scores and only scores higher than 30 were considered. Potential candidates were identified by comparison with supernatant without adiponectin and recombinant adiponectin control. Proteins found in these samples were excluded (colour) as well as proteins with low score even if not present in the controls (black). Only hit number 5 was unique to this sample with a high score of 63.1 and 1 peptide hit.

Hit Nb	Accession	Protein	MW [kDa]	Similar Proteins	Scores	Peptides	RMS90 [ppm]
1	MUC16_HUMAN	Mucin-16	2353.1	1	91.9 (M:91.9)	5	692.07
2	LDH6A_HUMAN	L-lactate dehydrogenase A-like 6A	36.5	4	57.6 (M:57.6)	2	185.09
3	LRP1_HUMAN	Prolow-density lipoprotein receptor-related protein 1	504.2	1	55.5 (M:55.5)	3	404.48
4	CSMD3_HUMAN	CUB and sushi domain-containing protein 3	405.7	1	55.1 (M:55.1)	3	204.18
5	KRA55_HUMAN	Keratin-associated protein 5-5	21.4	6	52.9 (M:52.9)	3	190.77
6	PPRC1_HUMAN	Peroxisome proliferator-activated receptor gamma coactivator-related protein 1	177.4	4	52.7 (M:52.7)	3	1297.58
53	MUC6_HUMAN	Mucin-6	252.0	1	36.5 (M:36.5)	2	996.31
246	PGBM_HUMAN	Basement membrane-specific heparan sulfate proteoglycan core protein	468.5	1	16.3 (M:16.3)	1	284.62

Table 7-2: Results of the Mascot database search on B cell supernatant with adiponectin

Data representative of the eight highest score detected after Mascot human protein database search which resulted in 303 hits. Proteins found in the adiponectin supernatant are represented in colour. At the bottom of the table, the hits found in the adiponectin supernatant but did not come with high score here were added. This sample did not contain hit number 5 found in the adiponectin supernatant and most of the proteins identified were also found in the recombinant adiponectin and adiponectin supernatant samples.

Hit Nb	Accession	Protein	MW [kDa]	Similar Proteins	Scores	Peptides	RMS90 [ppm]
1	MEG11_HUMAN	Multiple epidermal growth factor-like domains 11	110.8	2	102.7 (M:102.7)	5	518.82
2	SYNE2_HUMAN	Nesprin-2	795.9	1	67.9 (M:67.9)	4	641.57
3	MUC16_HUMAN	Mucin-16	2353.1	16	65.0 (M:65.0)	4	827.47
4	PI3R5_HUMAN	Phosphoinositide 3-kinase regulatory subunit 5	97.3	1	52.9 (M:52.9)	1	1081.17
8	CO7A1_HUMAN	Collagen alpha-1(VII) chain	295.0	1	49.0 (M:49.0)	2	368.63
18	KDM6B_HUMAN	Lysine-specific demethylase 6B	180.3	9	41.0 (M:41.0)	2	517.29
19	CO4A1_HUMAN	Collagen alpha-1(IV) chain	160.5	1	40.7 (M:40.7)	2	333.15
20	TITIN_HUMAN	Titin	3813.8	4	40.5 (M:40.5)	1	1663.60
60	LAMA1_HUMAN	Laminin subunit alpha-1	336.9	1	33.9 (M:33.9)	1	1158.36
101	WNK1_HUMAN	Serine/threonine-protein kinase WNK1 (95% homology WNK2)	250.6	1	21.9 (M:21.9)	1	74.32
29	LTBP1_HUMAN	Latent-transforming growth factor beta-binding protein 1	186.7	1	39.0 (M:39.0)	1	202

Table 7-3: Results of the Mascot database search on the recombinant adiponectin control

Data representative of the 11 of the highest scores detected after Mascot human protein database search which resulted in 264 hits. Proteins found in the adiponectin supernatant are represented in colour. At the bottom of the table, the hits found in the adiponectin supernatant but did not come with high score here were added. This sample did not contain hit number 5 found the adiponectin supernatant and most of the proteins identified were also found in the supernatants with and without adiponectin.

m/z	Elution time (min)	Score	Modification	Type	Association protein	Sequence
774.88	13.2	63.1	NA	CID	14-3-3 zeta/delta	SVTEQGAELSNEER

Table 7-4: Candidate peptide for Agent X

Protein database search on the ions detected in the Bs supernatant with adiponectin sample revealed the presence of the peptide presented in the table. This peptide originates from the 14-3-3 zeta/delta protein and will now be referred as PEPITEM.

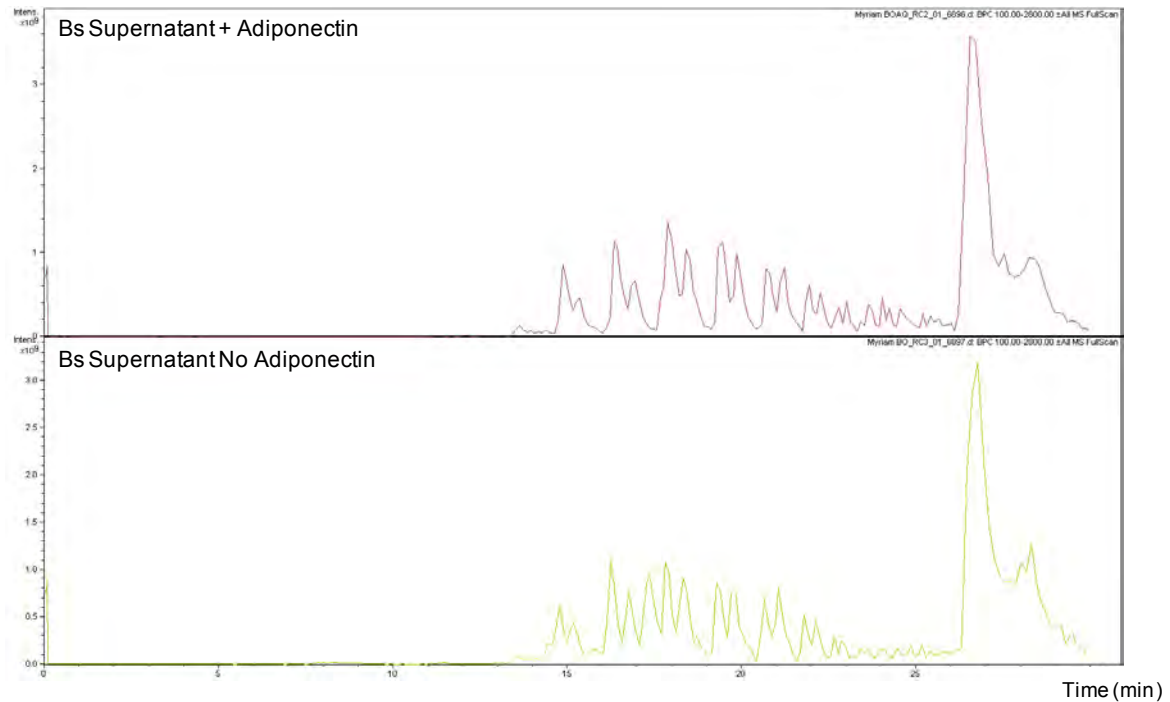


Figure 7-10: Comparison of the base peak chromatogram of B cell supernatant with or without adiponectin

Base peak chromatograms for the mass spectrometry analysis of the B cell supernatants with (top) or without (bottom) adiponectin. The chromatograms show high intensities peaks starting from about 14 minutes to 26 minutes that are likely to be contaminations. The large peak at the end shows the acetonitrile wash-up followed by re-equilibration of the column. Similar elution profiles are observed for both conditions.

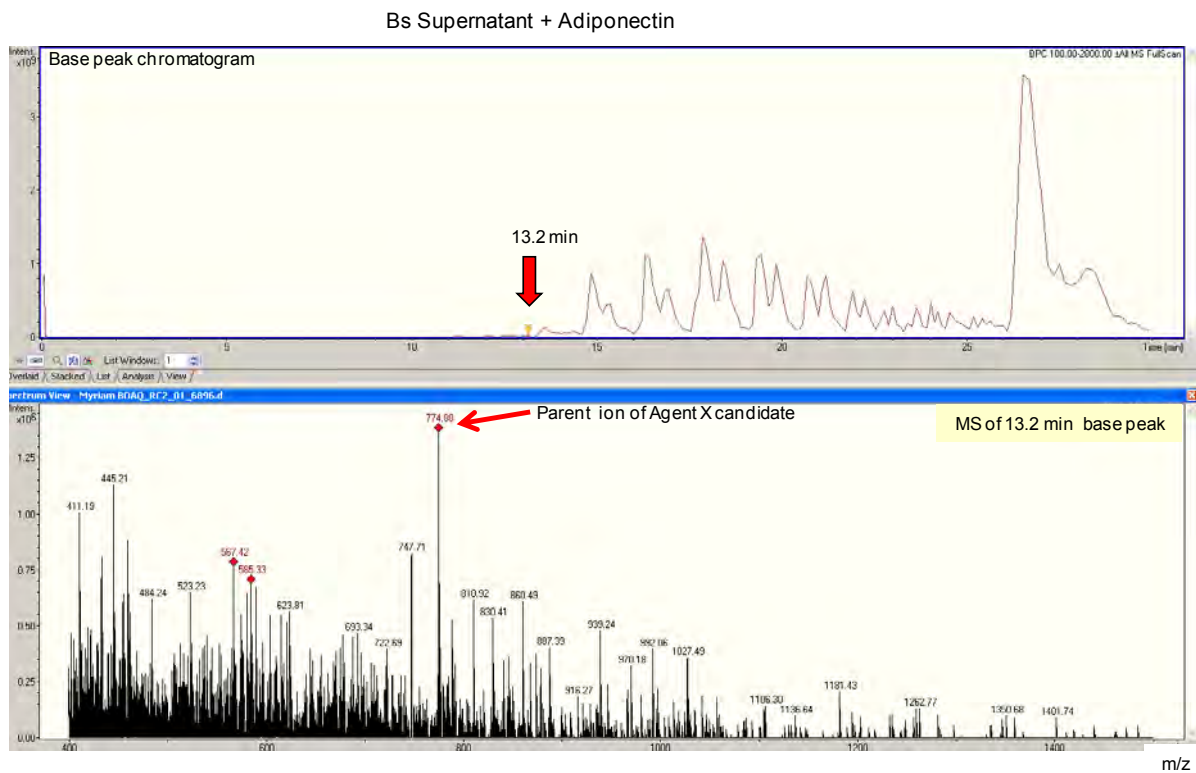


Figure 7-11: Base peak chromatogram of Bs supernatant with adiponectin and MS spectrum profile at 13.2 minutes

Protein database search identified the Agent X candidate at 13.2 min elution time on the base peak chromatogram (top). MS spectrum of the 13.2 min elution time point contains the parent ion of Agent X candidate with $m/z=774.88$ (bottom).

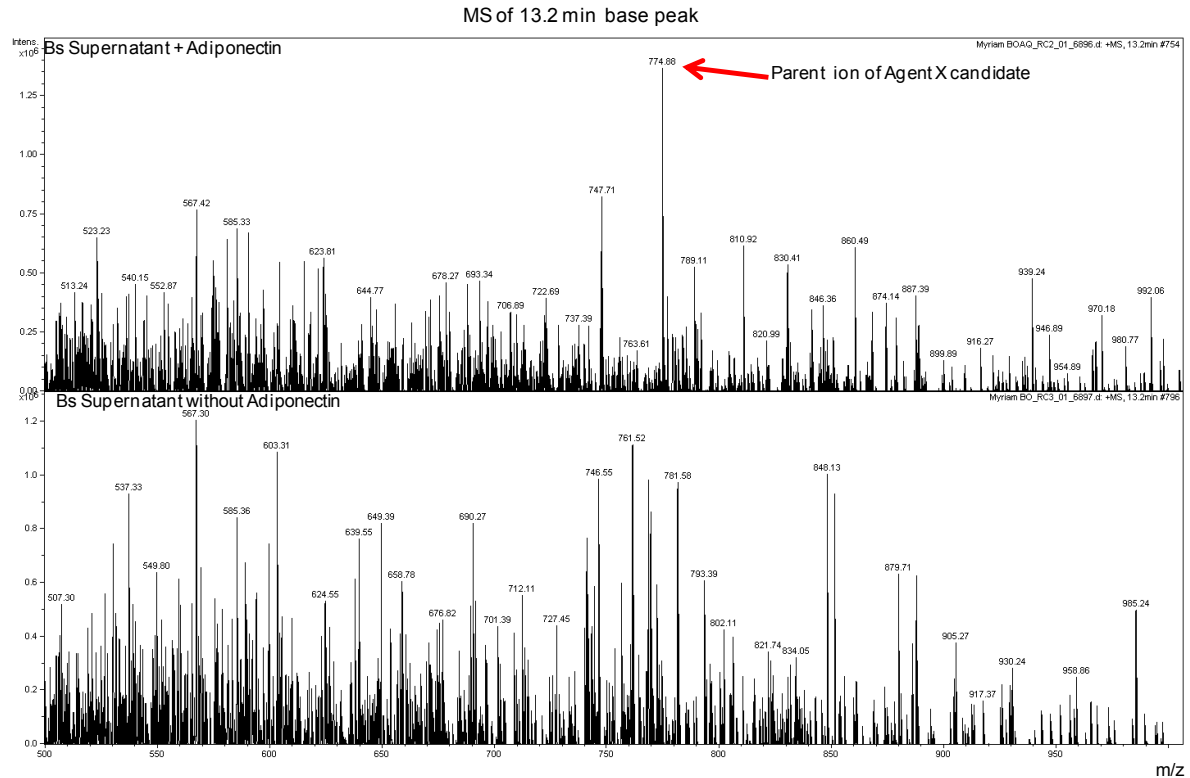


Figure7-12: Comparison of MS spectra from supernatant with or without adiponectin
 Comparison of the MS spectra from B cell supernatant with (top) or without (bottom) adiponectin at 13.2 min revealed that the parent ion for Agent X candidate at $m/z=774.88$ is only present in the supernatant of B cells treated with adiponectin.

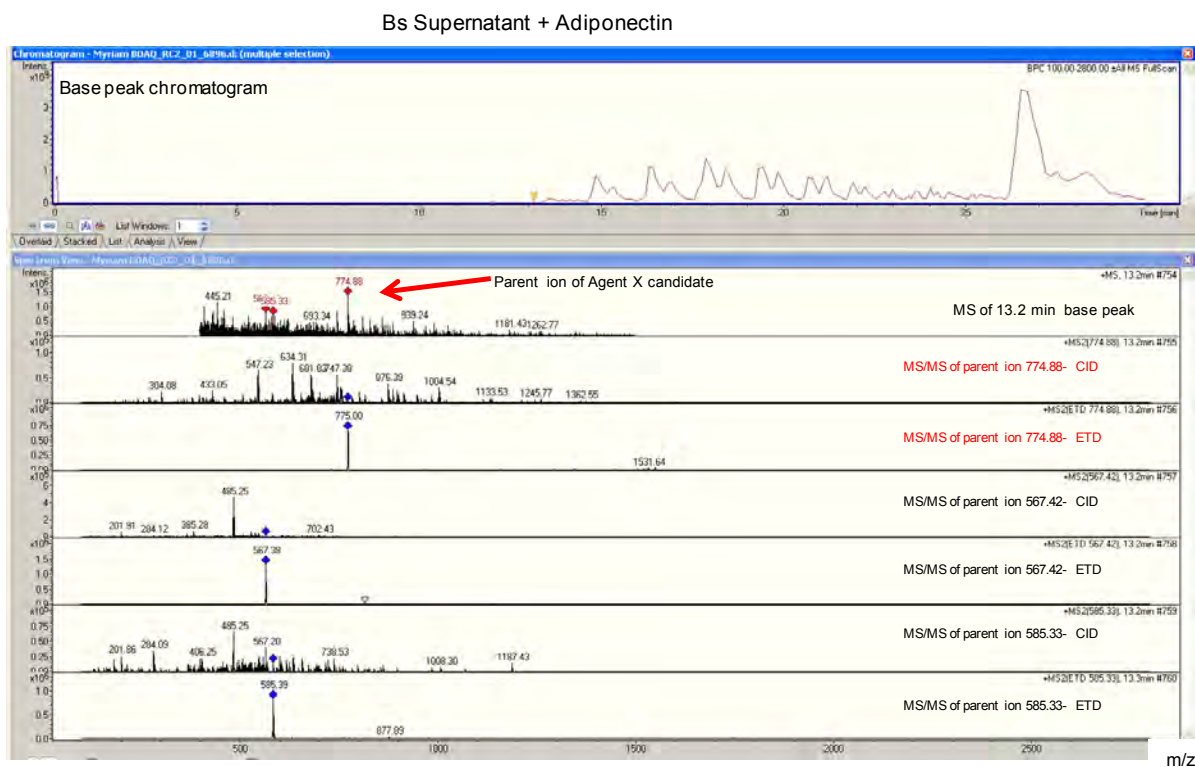


Figure 7-13: MS/MS of the Agent X candidate parent ion 774.88
MS/MS of the parent ion 774.88 was only possible by collision induced dissociation (CID) and not by electron transfer dissociation (ETD).

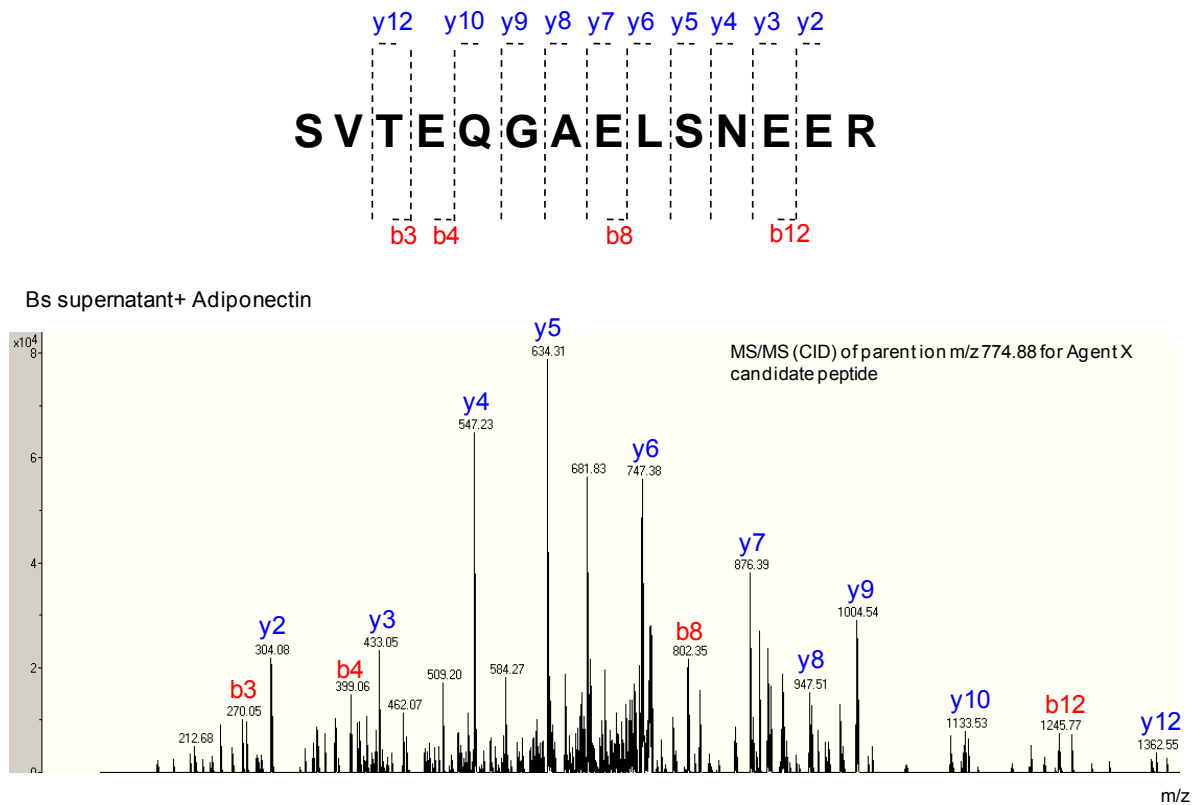


Figure 7-14: Determination of parent ion 774.88 sequence

The MS/MS spectrum is composed of b (fragmentation from N-terminal) and y (fragmentation from C-terminal) ions, which allow reconstituting the peptide sequence. Sequence of the standard was the same as the peptide identified in the supernatant of B cells treated with adiponectin.

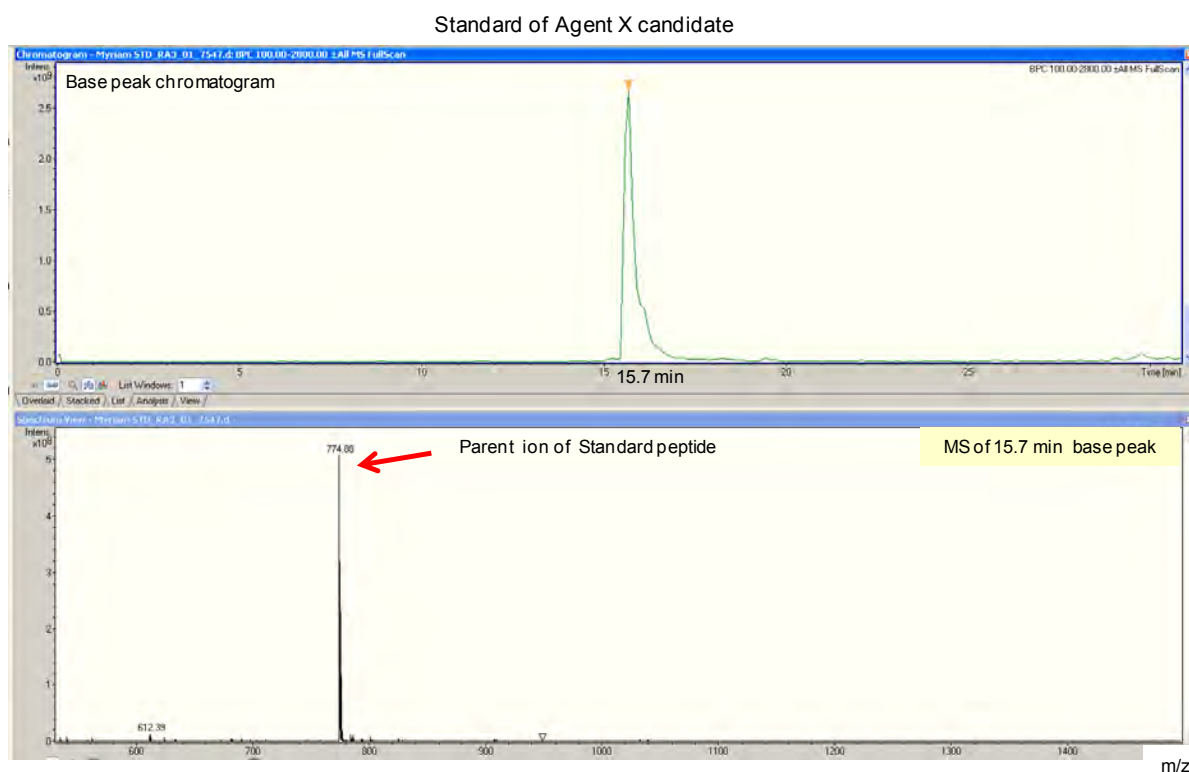


Figure 7-15: Base peak chromatogram and MS spectra (15.7 min) of the synthesised standard for the Agent X candidate

The Agent X candidate peptide was acquired and analysed by mass spectrometry. The base peak chromatogram shows high purity of the reagent as the elution one single high elution peak was found (top). The elution time of the standard is 15.7 minutes. This differs from the previous samples because of acetonitrile gradient changes in the analysis. In these conditions, the parent ion for the standard was identified at $m/z=774.88$, which is the same as the Agent X candidate in the B cell supernatant with adiponectin.

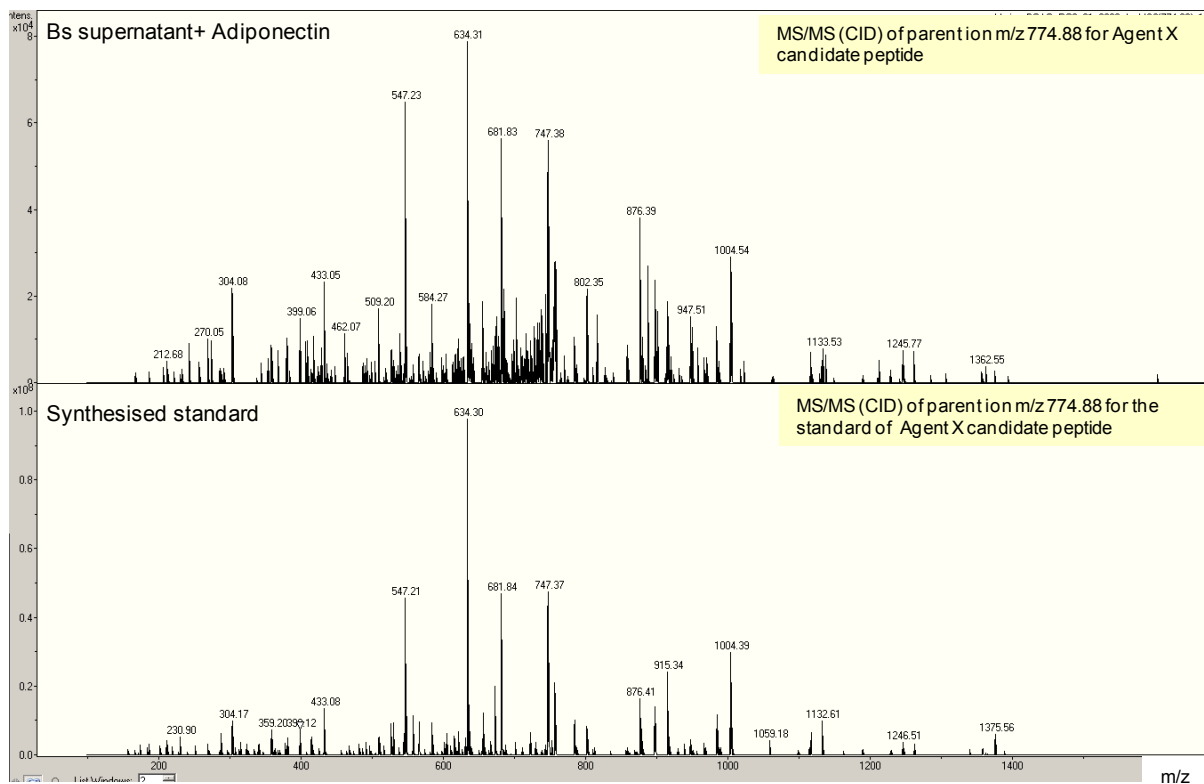


Figure 7-16: Comparison MS/MS parent ion m/z 774.88 from Bs supernatant with adiponectin to the synthesised peptide standard

MS/MS of the parent ion m/z 774.88 for the synthesised standard revealed the same fragments profile as the one in the B cell supernatant with adiponectin sample. This confirms that the sequence identified in the first analysis is correct.

2.4. Testing the biological function of PEPITEM

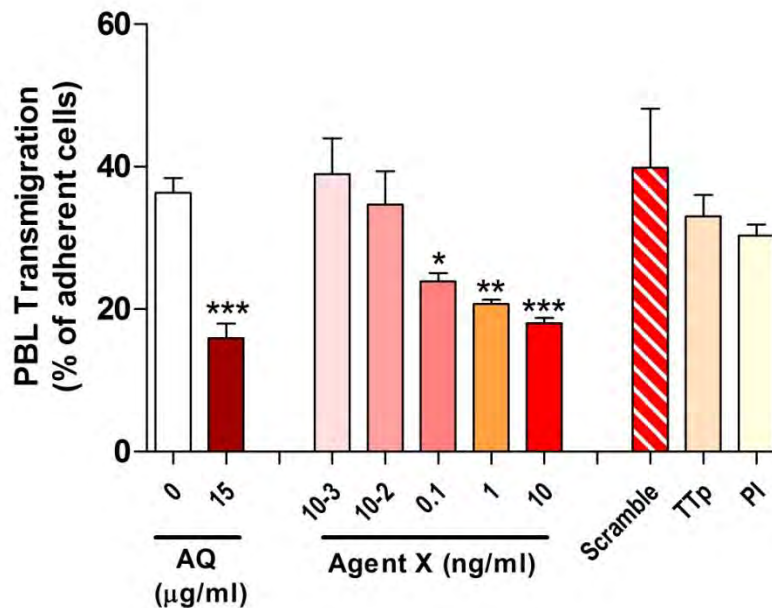
We wished to determine whether PEPITEM shows biological activity in our *in vitro* transmigration assay. We arranged for a synthesised peptide and scramble control to be synthesised by Alta Bioscience, University of Birmingham. Mass spectrometry of the peptide revealed high level of purity (**section 2.3**). PBL were isolated from peripheral blood and PEPITEM was added to the PBL at 0.001 to 10ng/ml just before addition of the PBL on the TNF- α /IFN- γ stimulated HUVEC. We observed a dose-dependent inhibition of PBL transmigration across the endothelium in presence of PEPITEM (**Figure 7-17a**). Importantly, treatment of PBL with the scramble control or tetanus toxoid and pro-insulin peptides did not inhibit PBL transmigration. These results indicate the specificity of PEPITEM. The peptide had an EC₅₀ of 18.6pM in these experiments, calculated using non linear regression analysis (**Figure 7-17b**). The R² of the curve fitting to the data is 0.77, which indicates the goodness of the non linear regression fit is close to 1.

Finally, we investigated the biological activity of PEPITEM *in vivo* using the B cell KO model of peritoneal inflammation. B cells KO mice were intravenously injected with 100 μ g PEPITEM or the scramble control four hours before intraperitoneal injection of zymosan which also contained 100 μ g of the relevant peptide. After 24 hours, a final intravenous injection of 100 μ g of peptide was given. Thus the mice received a total of 300 μ g of the PEPITEM or scramble peptides. Both peptides were synthesised with an additional amide group on C-terminal. The amide group provides protection of the peptide from proteolytic cleavage that might occur *in vivo*. After 48 hours, peritoneal leukocytes were collected and counted by flow cytometry. We detected a significant reduction of CD3⁺ T cells recruitment in the inflamed peritoneum of B cell KO mice treated with PEPITEM (**Figure 7-18a**). Importantly, this was not observed with the scramble peptide treatment confirming the specificity of the peptide. In addition, analysis of T cell subsets revealed less recruitment of

Chapter 7- A novel adiponectin-induced, B cell-derived peptide, PEPITEM, inhibits the migration of T cells CD4⁺ T cells (**Figure 7-18b**), especially of the effector memory subset, which was the major subset recruited in the B cell KO without peptide treatment (**Figure 7-18c**).

In conclusion, the peptide identified as the B cell-secreted-Agent X inhibits lymphocytes transmigration both *in vitro* and *in vivo*. These data strongly support a biological role for Agent X, henceforth referred to as **PEP**tide **I**nhibitor of **T**rans-**E**ndothelial **M**igration (**PEPITEM**).

a)



b)

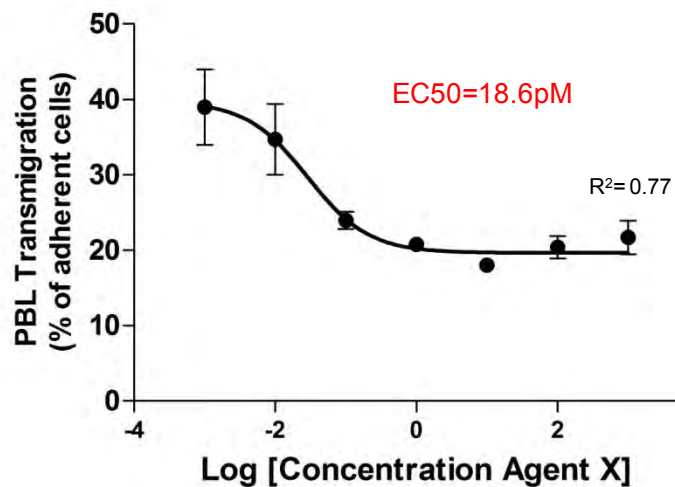


Figure 7-17: Agent X inhibits PBL transmigration

(a) PBL were isolated from peripheral blood and transmigration assays were performed using TNF- α /IFN- γ stimulated HUVEC and adiponectin pre-treated whole PBL, whole PBL containing Agent X at 0.001 to 10 ng/ml, whole PBL containing 10 ng/ml of Agent X scramble, PBL with tetanus toxoid peptide (TTP) at 10 ng/ml and PBL with pro-insulin (PI) at 10 ng/ml. PBL transmigration was dose-dependently reduced in presence of Agent X but not with the scramble, TTP and PI controls. Data are a pool of three independent experiments and were analysed using one-way ANOVA and Bonferroni's multiple comparison post test. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. (b) An EC₅₀ of 18.6 pM was calculated using non linear regression analysis.

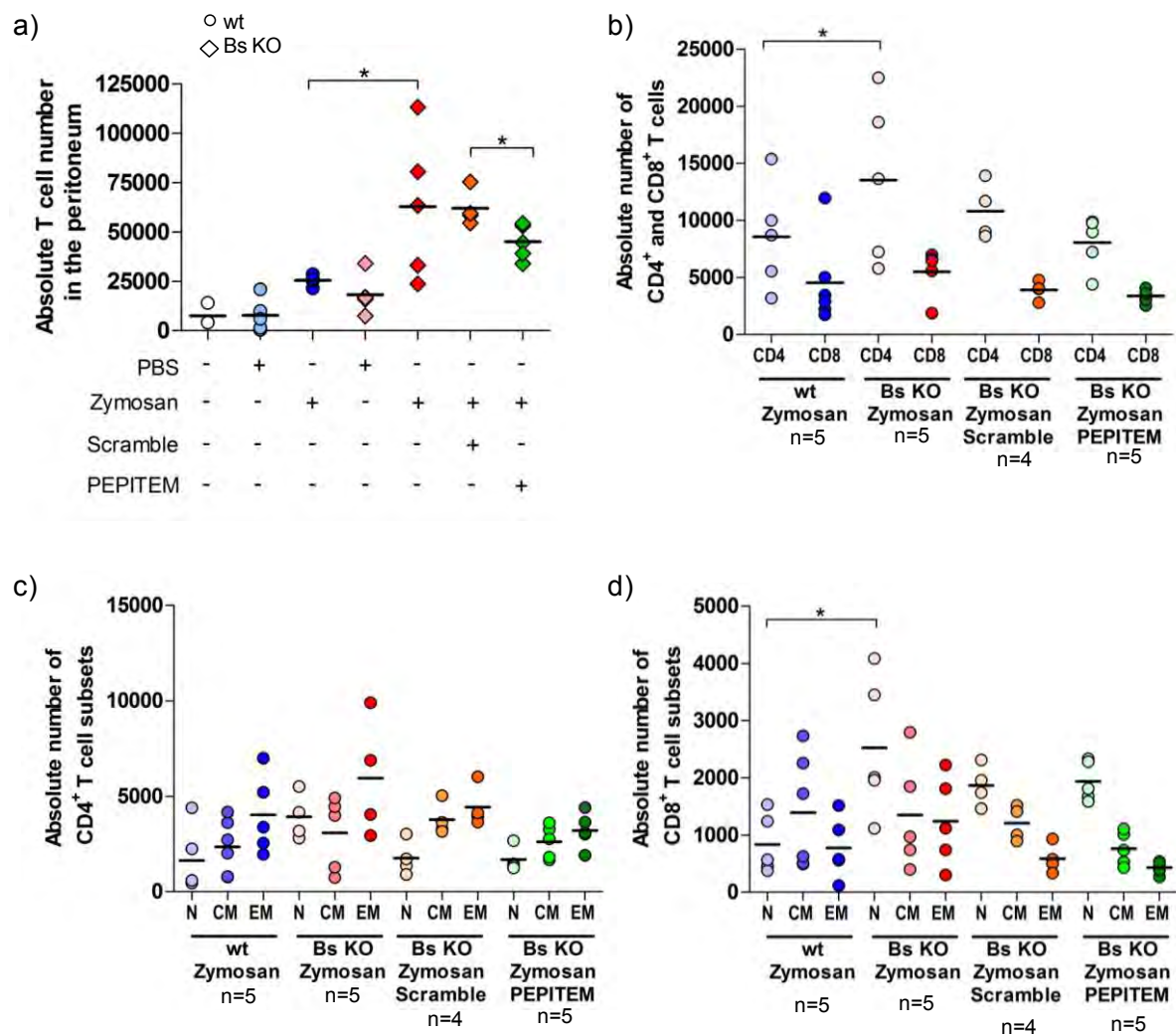


Figure 7-18: PEPITEM reduces T cell recruitment *in vivo*

Leukocytes were collected from the peritoneum after 48 hours with zymosan injection in the wt or B cell knock-out. T cells were identified by expression of CD3 (a). CD4⁺ and CD8⁺ T cells were identified by gating on CD3 first and by the expression of CD4 and CD8 (b). CD3⁺ and CD4⁺ (c) or CD8⁺ (d) T cells were first gated and naive (N), central memory (CM) and effector memory (EM) T cells were identified by the expression of CD44 and CD62L. Naive CD4⁺ are CD62L⁺CD44⁻, central memory T cells are CD62L⁺CD44⁺ and effector memory T cells are CD62L⁻CD44⁺. Data for each mouse groups were pooled, represented as mean and were analysed using one-way ANOVA and Bonferroni's multiple comparisons post-test. *p≤0.05.

3. Discussion

In this chapter, we demonstrate a novel mechanism of action of adiponectin which mediates inhibition of lymphocyte trans-endothelial migration. We show that under conditions of adiponectin stimulation, B cells generate a newly identified soluble peptide referred to as PEPITEM, which is able to promote release of S1P by the endothelium, thus affecting T cell transmigration (**Figure 7-19**). We have confirmed these findings *in vivo* in the B cell knock-out mouse using a peritoneal model of inflammation.

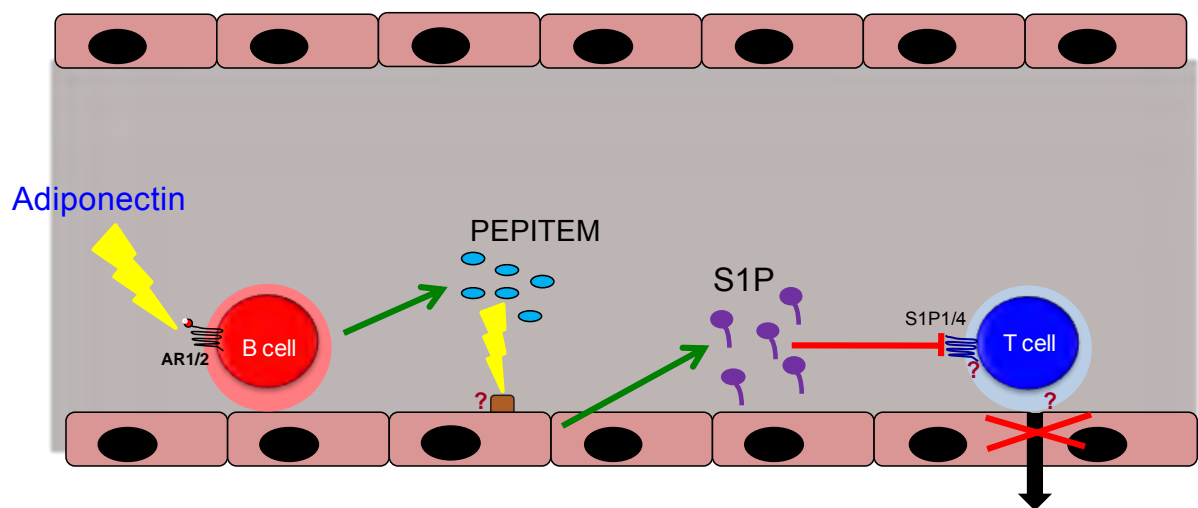


Figure 7-19: Mechanism of action of adiponectin-mediated inhibition of lymphocyte trans-endothelial migration

Adiponectin stimulates production of PEPITEM by B cells. PEPITEM triggers the secretion of S1P by the endothelium, theoretically by interaction with its receptor on the endothelium. S1P interaction with its receptor on the T cells inhibits their transmigration. S1PR signalling and downstream effects on migration are unknown in this context.

- **Involvement of S1P in the mechanism of action of adiponectin**

Here, we have determined that B cell modulate T cell migration through a secreted agent under adiponectin stimulation. In addition, we demonstrate that S1P inhibits T cell migration through macrovascular endothelium. Although S1P has been widely associated with inducing T cell egress from secondary lymphoid organs into efferent lymphatics, recent reports reveal a capacity for S1P to inhibit T cell migration from tissues to afferent lymphatics (Legerwood *et al.*, 2008). This is mediated by high S1P levels in tissues during inflammation (Legerwood *et al.*, 2008; Roviezzo *et al.*, 2011). These studies show the importance of S1P and S1PR signalling during an inflammatory response and highlight localised effects which differ in lymph nodes and in the periphery. Functions in the periphery are believed to help T cells accomplish their functions at the site of injury, as they cannot migrate to the lymph nodes and are retained in tissues. Here we report for the first time a role for S1P in regulating T cell migration in the vascular compartment. We believe that this data is consistent with observations of S1P mediated regulation in other compartments, indeed previous reports of S1P function in regard of T cell migration made this an obvious candidate for regulating T cell function in our assays and it is therefore not surprising that we observe a S1P-induced inhibition of T cell migration in our model. Interestingly, the functional range of concentrations in our experiments is in agreement with published efficacy of this agent to modulate T cell function (Legerwood *et al.*, 2008; Roviezzo *et al.*, 2011). We used in the order of 0.1 μ M to induce an inhibition of lymphocyte transmigration. However, the data measuring S1P concentrations *ex vivo* and *in vivo* show variations between tissue compartments and some differences between studies. In human serum, S1P levels range from 0.4 to 1.1 μ M (Caligan *et al.*, 2000; Berdyshev *et al.*, 2005). In plasma, circulating concentrations of S1P have been reported between 0.2 to 0.9 μ M with the majority bound to albumin and lipoproteins (Murata *et al.*, 2000). Notably however, S1P bound to lipoproteins

is still active (Nofer *et al.*, 2004). S1P concentrations are lower in the lymph (80nM) and in the lymph nodes and other tissues (5-20nM) (Cyster, 2005). In mice, some studies show high circulating S1P levels of about 1 to 10 μ M and about 100nM in the lymph (Pappu *et al.*, 2007). Although we used a “high” concentration of S1P to observe an effect on T cell migration, this was still close to the circulating levels, suggesting these concentrations were appropriate in the vascular compartment. It is also possible that S1P levels become high locally. This would mean that higher global S1P levels would be required to see an effect. Indeed, we believe that B cells that adhere on the endothelium and secrete PEPITEM will induce a localised rise in S1P release by the endothelium.

Interestingly, we observed that the inhibitory effects of exogenous and endogenous S1P were lost over time. We associated this with the short half-life of S1P in plasma, which has been reported at 15 minutes (Venkataraman *et al.*, 2008). Gratifyingly, the dynamics of S1P mediated inhibition of T cell migration were the same whether S1P was induced through addition of adiponectin, or was added as a synthetic exogenous agent. We believe that these similar profiles are indicative of the close relationship between adiponectin signalling and S1P production and therefore confirm our hypothesis that S1P is involved in the adiponectin-mediated inhibition of lymphocyte trans-endothelial migration. In addition, when we used a S1PR antagonist, we lost the inhibitory effect of adiponectin. Collectively, these data show the involvement of S1P in the adiponectin mediated inhibition of T cell migration

It should be noted that S1P was such an obvious choice of agent for regulating T cell migration that initially we thought it might have been generated by B cells in response to adiponectin stimulation. However, experiments measuring SPHK1 and 2 in B cells, which lacked these kinases, refuted this hypothesis. These observations are in conflict with one other study which found expression of SPHK in B lymphoblastoid cells (Pi *et al.*, 2006). However,

in this study they did not compare the gene expression to other positive controls and used an immortalised cell line which is likely to have different transcriptional characteristics compared to primary B cells. SPHKs are essential for S1P synthesis. Indeed, SPHK-deficient mice have no detectable S1P in the plasma or lymph (Pappu *et al.*, 2007). We observed high expression of both SPHKs in HUVEC. High expression of SPHKs has been reported before in HEV, but to our knowledge, the current literature does not report their expression in other vascular beds (Pham *et al.*, 2010). This finding led us to the hypothesis that S1P was synthesised by the HUVEC in our system, under the influence of a B cell-secreted agent that we have now identified as PEPITEM.

We sought to link the S1P mediated effects of adiponectin to HUVEC by depleting SPHKs in these cells using siRNA. However, SPHK1 was highly up-regulated upon TNF- α and IFN- γ stimulation of HUVEC and we had great difficulty in achieving suitable levels of depletion. This was still the case after multiple transfections of the siRNA duplexes. Therefore, we attributed the lack of effect of siRNA to this suboptimal knock-down and not to the fact that the kinases were irrelevant to our system. Although the antagonist data confirm the involvement of S1PR and therefore S1P in the actions of adiponectin, this still needs to be confirmed with more stringent experimental approaches. It would be relevant to use lentiviral particles expressing siRNA against SPHKs, as this would provide a more efficient and permanent knock-down. An easier way would be to inhibit SPHKs activity using chemical compounds such as L-cycloserine or DL-threo-dihydrosphingosine (DTD) (Roviezzo *et al.*, 2011). Additionally, a S1P blocking antibody, referred as sphingomab, exists (Takabe *et al.*, 2008). The other alternative would be to measure S1P levels in the media after stimulation with PEPITEM using mass spectrometry (Caligan *et al.*, 2000; Berdyshev *et al.*, 2005) and relate these to function. In the future a combination of these strategies would need to be used to deliver a concrete proof of hypothesis.

In agreement with the literature, we found very low levels of S1PR receptor expression on the extracellular membranes of T cell (Cyster, 2005). It may seem unusual that antagonism of a small quantity of receptors is enough to ablate S1P signalling in these cells, however, we did not investigate the biology of the receptors in presence of the antagonist, S1P, adiponectin or PEPITEM. It would be interesting to study the expression profile on the T cell surface in presence of these, and moreover on the firmly adhered or transmigrated PBL, as we have only conducted analysis on freshly isolated cells in suspension. Indeed, we do not know if in our model the receptor signalling is regulating migration, for example by modifying integrin function, or if S1P is causing receptors internalisation and therefore blocking a signal essential for trans-endothelial migration.

Finally, it would be interesting to treat the endothelium with S1P and see if the effect is still observed, as evidences is available showing that EC express S1P receptors for S1P and these can be internalised upon agonist binding. Functions for S1P in EC include regulation of EC-EC junctions (Singer *et al.*, 2005) and this may be relevant to the mechanism of action of adiponectin in our assay as most lymphocytes migrate between the endothelial cells. Indeed, this is an important experiment as those conducted to date do not discriminate between the effects of S1P on T cells and HUVEC.

Regarding a mechanism of action for S1P on T cell transmigration, it has been shown that in secondary lymphoid organs, S1P induces S1PR1 signalling that overcomes CCR7-mediated retention of T cells, thereby allowing egress into the circulation (Pham *et al.*, 2008). In the periphery, no mechanism for the function of S1P has so far been identified. We propose a potential involvement of cyclic AMP (cAMP) in our model. cAMP has inhibitory effects on leukocytes trans-endothelial migration (Lorenowicz *et al.*, 2007). The mechanism of cAMP action on lymphocyte transmigration is still unknown but cAMP can also block adhesion by reducing the activation of VLA-4 ($\alpha_4\beta_1$) (Lorenowicz *et al.*, 2007). We believe that action on

integrin activation could be a potential mechanism by which S1P could modulate T cell transmigration. In addition, studies have now shown that S1P is able to stimulate cAMP production by muscle cells, and that adiponectin is able to induce its production in endothelial cells (Damirin *et al.*, 2005; Ouchi *et al.*, 2000).

- **Identification of PEPITEM**

Proteomics is a widely used technique to identify active compounds in supernatants. However, mass spectrometry analysis detects a considerable amount of proteins/peptides, especially when using a non-tryptic approach. Indeed a lot of the peptides found using this approach are either false positives or the result of contamination. Problems with contaminations are a recurrent issue in mass spectrometry especially when using complex biological samples. To prevent this, the columns are intensively washed in between samples and no sample will be processed until the system is clean. To eliminate false positives, it is essential to have the right negative controls. In our case, we compared supernatants from B cells stimulated with adiponectin with supernatants from untreated cells. Moreover these supernatants were generated in serum/protein free medium to reduce the complexity of the mixtures being analysed. Using this strategy, contamination and false positives can be excluded by simple comparison between the conditions. To further improve our samples purity, we pre-purified them on a C18 column. The conditions of washes and elution allowed us to enrich the samples for small molecules such as peptides and to exclude large proteins that irreversibly bind to the column.

Using this technique, we identified PEPITEM, a 14 amino acids peptide belonging to the 14-3-3 zeta/delta protein. The identification resulted in a very high score indicating the accuracy of the match between the peptide in the sample and the peptide in the databases. In addition, the peptide was specific to the adiponectin-stimulated B cell supernatant and the

peptide in this sample has similar profile of MS and MS/MS to the synthetic standard that we had manufactured. Together these allow us to have great confidence in the identity and specificity of PEPITEM.

14-3-3 proteins are found in all eukaryotic organisms with high evolutionary conservation (**Figure 7-20**), often in multiple isoforms, from 2 in yeast, nematode and drosophila to 13 in plants and regulate a whole range of biological processes. In the tomato, 14-3-3 proteins regulate MAP kinases pathway, ion channels and lipoxygenases that are important for mechanisms of defence (Oh *et al.*, 2010). In yeast, 14-3-3 proteins have a major role in the subcellular localization of proteins. For instance, Yak1 protein kinase, involved in glucose-sensing, associates with cytoplasmic 14-3-3 proteins in the absence of glucose, whereas in the presence of glucose it is mainly present in the nucleus (van Heusden, Steensman, 2006). Interestingly 14-3-3 proteins regulate the life span of *Caenorhabditis elegans* (nematode) by cooperating with important transcription factors such as SIR-2.1 and DAF-16/FOXO (Wang *et al.*, 2006).

In mammals, the 14-3-3 proteins are a family of seven highly homologous and conserved isoforms named β (beta), ϵ (epsilon), γ (gamma), η (eta), σ (sigma), τ (tau), ζ (zeta) depending on their elution profiles. The 14-3-3 proteins named α (alpha) and δ (delta) are the phosphorylated versions of β and ζ respectively (reviewed in Fu *et al.*, 2000). The proteins are expressed in almost all tissues (Celis *et al.*, 1990). 14-3-3 proteins have been reported to bind to and modulate the function of a wide number of intracellular proteins (Fu *et al.*, 2000). Binding to their partners results in inhibition or stimulation of the partner protein. Therefore, these proteins are involved in the regulation of diverse biological processes such as metabolism, cell cycle regulation, apoptosis and protein trafficking (Fu *et al.*, 2000; Bridges *et al.*, 2005; Dougherty *et al.*, 2004). 14-3-3 proteins essentially interact with their partners through a phosphorylated serine motif (Muslin *et al.*, 1996). Binding of 14-3-3 proteins

mediates changes in signalling capacities of their multiple ligands (**examples in Figure 7-21**). For instance, binding of 14.3.3 ζ to Raf-1 modulates its conformation, while binding of 14.3.3 β to lip35 allows exit of MHC class II from the endoplasmic reticulum, as a final example, interaction of 14-3-3 τ with PKC μ inhibits its kinase activity (Morrison *et al.*, 1997; Kuwana *et al.*, 1998; Hausser *et al.*, 1999). Disruption of the expression and/or function of 14-3-3 proteins can therefore cause clinical disorders. Although, 14-3-3 proteins have not been associated with a specific disease, they have been connected with various pathological processes. For instance, many of the 14-3-3 partner proteins are products of oncogenes, which suggest an involvement of 14-3-3 proteins in cancer (Lodygin *et al.*, 2005). In addition, 14-3-3 proteins are also highly expressed in the brain and aberrant regulation of expression or function is implicated in neurological disorders (Layfield *et al.*, 1996).

To our knowledge, none of the seven 14-3-3 isoforms have been directly linked to the regulation of leukocyte trafficking *in vitro* or *in vivo*. However, some reports show involvement of 14-3-3 beta and zeta/delta in regulation of cell spreading (reviewed in Mhaweche, 2005). It seems that binding of 14-3-3 beta to $\beta 1$ integrin helps cell spreading and migration (Han *et al.*, 2001; Rodriguez, Guan, 2005). 14-3-3 zeta/delta acts on integrin signalling by binding to Cas, an integrin signalling adaptor (Garcia-Guzman *et al.*, 1999). These observations were reported in fibroblast cell lines as well as in T cells (Nurmi *et al.*, 2006). In addition, 14-3-3 proteins were found to interact with apelin-13 in HUVEC causing an increase of monocytes adhesion (Li *et al.*, 2010). However, this study has limitations as the authors used the THP-1 monocytic cell line which might not behave like freshly isolated primary monocytes and also measured adhesion on ECV304 endothelial cells, which are not considered HUVEC anymore. The data in this area is therefore limited and need more investigations.

Interestingly, although 14-3-3 zeta/delta expression has been measured in almost all leukocytes subsets (i.e. monocytes, CD4⁺ and CD8⁺ T cells, B cells and NK cells (BioGPS), 14-3-3 proteins have not been closely associated to the function of the immune system. The only reports potentially involving 14-3-3 proteins in the immune function, describe their binding capacity to mediators of T cell activation. Binding of 14-3-3 proteins to different intermediate in the T cell stimulation pathway induces stimulation or inhibition of T cells activation (Di Bartolo *et al.*, 2007; Liu *et al.*, 1996; Meller *et al.*, 1996). For instance, the interaction of 14-3-3 τ with Cbl and phosphatidylinositol 3-kinase triggers T cell activation (Liu *et al.*, 1996). In addition, 14-3-3 ϵ and ζ cooperates with SPL-76 to tune T cell activation (Di Bartolo *et al.*, 2007). On the other hand, binding of 14-3-3 τ with PKC inhibits T cell activation (Meller *et al.*, 1996). However, the data available on this topic is limited and sometimes controversial. Certainly there is no indication that active peptides of these proteins play an integral role in regulation of immune function.

PEPITEM is specific to 14-3-3 zeta/delta as evidenced by a comprehensive protein blast (NCBI) search. Indeed, although other 14-3-3 proteins have similar sequences, none is an absolute match for PEPITEM. However and very interestingly, the PEPITEM sequence in 14-3-3 zeta/delta is highly conserved between species. It is found in a majority of mammals, i.e. mice, rats, panda and horse but also birds (chicken), at 100% sequence homology (**Figure 7-22**). Moreover a highly conserved sequence (92-93% homology) is present in fish and amphibians. Remarkably, a sequence recognisable as PEPITEM is also present in nematodes (92% homology). Two types of 14-3-3 are also found in *Saccharomyces cerevisiae* (yeast) and include a sequence close to PEPITEM but with more differences compared to other species (78% homology) and lack some of the sequence and similarly for insects and plants (73% homology).

PEPITEM is located from residue 28 to 41 in the 14-3-3 zeta/delta protein. Several studies have determined the crystal structure of 14-3-3 zeta/delta on its own, in dimers or interacting with its ligands (Liu *et al.*, 1995; Ottmann *et al.*, 2007). A 14-3-3 zeta/delta monomer is composed of nine anti-parallel α -helices forming the limit of the amphiphile groove allowing interaction with its partners (Liu *et al.*, 1995). This structure is conserved between vertebrates, yeast and even plants (Liu *et al.*, 1995). We positioned PEPITEM on the 14-3-3 zeta/delta crystal structure and it is located between α -helice2 and α -helice3, with some residues forming the start of each helix (**Figure 7-22**). In addition, we noticed that PEPITEM is not cryptic, instead it is quite accessible and exposed at the protein outer surface. We assume that this is essential for proteolytic cleavage from the parent molecule.

This peptide has never been identified before except as a tryptic peptide for the recognition of the 14-3-3zeta/delta protein. The only other occurrence of PEPITEM in the literature, involves the same sequence as a peptide presented by HLA-DQ8 in the NOD mice (Suri *et al.*, 2005). But in this reference there is no indication of a physiological role for the peptide.

Finally, we confirmed the capacity of PEPITEM to inhibit T cell migration in our *in vitro* assay but also *in vivo*. The peritoneal model of inflammation previously allowed us to show that absence of B cell during acute inflammation promotes higher recruitment of T cells in the peritoneum. And this can be partially reversed when PEPITEM is administrated to the B cell KO mice. For this experiment, we acquired PEPITEM with an additional amide group on C-terminal, as this is known to protect against proteolytic cleavage. As we have not yet investigated the pharmacokinetics of PEPITEM *in vivo*, we had no indication of dose of peptide to administer. We believe that this explains the suboptimal effects of PEPITEM administration in the peritonitis model (i.e. about 40% of maximal effect was induced by our protocol). It would be interesting to investigate PEPITEM in models of T cell-mediated

Chapter 7- A novel adiponectin-induced, B cell-derived peptide, PEPITEM, inhibits the migration of T cells disease, such as T1D, atherosclerosis, rheumatoid arthritis, Crohn's disease and other autoimmune and/or chronic inflammatory diseases. However, it is essential to conduct pharmacokinetic studies on the peptide first in order to optimise appropriate doses in the animal models.

- **Conclusions**

To summarize, we provide evidences for a novel role for B cells in regulating T cell recruitment in the presence of adiponectin. We identified a unique peptide secreted by B cells under adiponectin stimulation. We demonstrate that this peptide is regulating T cell migration by stimulating S1P release from the endothelium (**Figure 7-19**). These effects were validated *in vivo*, using a model of inflammation in the B cell knock-out mice. This small peptide is an interesting potential therapeutic agent in T cell-mediated diseases. Whilst this work is innovative, it leads to a number of important mechanistic and therapeutic investigations that need to be explored.

<i>Homo sapiens</i>	MDKNELVQKAKLAEQAERYDDMAACMKSVTEQGAELSNEERNLLSVAYKNVVGARRSSWRVV	
<i>Mus musculus</i>	MDKNELVQKAKLAEQAERYDDMAACMKSVTEQGAELSNEERNLLSVAYKNVVGARRSSWRVV	
<i>Gallus gallus</i>	MDKNELVQKAKLAEQAERYDDMAACMKSVTEQGAELSNEERNLLSVAYKNVVGARRSSWRVV	
<i>Danio rerio</i>	MDKLELQKAKLAEQAERYDDMAACMKVTEQGEELSNEERNLLSVAYKNVVGARRSAWRVI	
<i>Xenopus laevis</i>	MDKNELVQKAKLAEQAERYDDMAACMKVTEEGEELSNEERNLLSVAYKNVVGARRSSWRVV	
<i>Drosophila</i>	VDKEELVQKAKLAEQSERYDDMAQAMKSVTEITGVELSNEERNLLSVAYKNVVGARRSSWRVI	
<i>A. thaliana</i>	RDEFVYMAKLAEQAERYEEMVEFMKVAKAVDKDELTVVEERNLLSVAYKNVIGARRASWR	
<i>C.elegans</i>	KEELVNRKAKLAEQAERYDDMAASMKVTELGAELSNEERNLLSVAYKNVVGARRSSWRVI	
<i>S.cerevisae</i>	AKLAEQAERYEEMVENMKAVASSGQELSVEERNLLSVAYKNVIGARRASWRIV	
<i>Homo sapiens</i>	SSIEQKTEGAEEKQQMAREYREKIETELRDICNDVLSLLEKFLIPNASQAESKVFYLMKMGD	
<i>Mus musculus</i>	SSIEQKTEGAEEKQQMAREYREKIETELRDICNDVLSLLEKFLIPNASQESKVFYLMKMGD	
<i>Gallus gallus</i>	SSIEQKTEGAEEKQQMAREYREKIETELRDICNDVLSLLEKFLIPNASQAESKVFYLMKMGD	
<i>Danio rerio</i>	SSIEQKTEGNDKKLQMVKEYREKVEGELRDICNEVLTLLGKYLIKNSTNSESKVFYLMKMGD	
<i>Xenopus laevis</i>	SSIEQKTEGAEEKQEMSREYREKIEAELREICNDVNLNLLDKFLIANATQESKVFYLMKMGD	
<i>Drosophila</i>	SSIEQKTEASARKQQLAREYREVEKELREICYEVLGLLDKYLIPKASNPEKVFYLMKMGD	
<i>A. thaliana</i>	SSIEQKEESRGNDHVSILRDYRSKIETELSDICDGLIKLLDTILVPAASGDSKVFYLMKMGD	
<i>C.elegans</i>	SSIEQKTEGSEKKQMAKEYREKVEKELRDICQDVLNLLDKFLIPKAGAAESKVFYLMKMGD	
<i>S.cerevisae</i>	SSIEQKEESKEKSEHQVELIRSYRSKIETELTKISDDILSVLDSHLIPSSATTGESKVFYLMKMGD	
<i>Homo sapiens</i>	YYRYLAEVAAGDDKKGIVDQSQQAYQEAFAISKEMQPTHPIRLGLALNFSVFYYEILNSPE	
<i>Mus musculus</i>	YYRYLAEVAAGDDKKGIVDQSQQAYQEAFAISKEMQPTHPIRLGLALNFSVFYYEILNSPE	
<i>Gallus gallus</i>	YYRYLAEVAAGDDKKGIVEQSQQAYQEAFAISKEMQPTHPIRLGLALNFSVFYYEILNSPE	
<i>Danio rerio</i>	YYRYLAEVAAAADDKMDTITNSQAGAYQDAFEISKMDQPTHPIRLGLALNFSVFYYEILNSPEQ	
<i>Xenopus laevis</i>	YYRYLAEVAAGDAKSDIVGQSQKAYQDAFDISKTEMQPTHPIRLGLALNFSVFYYEILNCPD	
<i>Drosophila</i>	YYRYLAEVATGDARNTVDDSQAYQDAFDISKGMQPTHPIRLGLALNFSVFYYEILNSPD	
<i>A. thaliana</i>	YHRYLAEFKSGQERKDAAEHTLTAYKAAQDIANSELPATHPIRLGLALNFSVFYYEILNSPD	
<i>C.elegans</i>	YYRYLAEVASGDDRNSVVEKSQSSYQEAFDIAKDKMQPTHPIRLGLALNFSVFYYEILNAPD	
<i>S.cerevisae</i>	YHRYLAEFSSGDAREKATNSSLEAYKTASEIATTELPPTHPIRLGLALNFSVFYYEIQNSPD	
<i>Homo sapiens</i>	KACSLAKTAFDEAIAELDTLSEESYKDSLIMQLLRDNLTLWTSDTQGDEAEAGEGGEN	
<i>Mus musculus</i>	KACSLAKTAFDEAIAELDTLSEESYKDSLIMQLLRDNLTLWTSDTQGDEAEAGEGGEN	99%
<i>Gallus gallus</i>	KACSLAKTAFDEAIAELDTLSEESYKDSLIMQLLRDNLTLWTSDTQGDEAEAGEGGEN	99%
<i>Danio rerio</i>	ACSLAKQAFDEAIAELDTLNEDSYKDSLIMQLLRDNLTLWTSD	84%
<i>Xenopus laevis</i>	KACSLAKTAFDEAIAELDTLSEESYKDSLIMQLLRDNLTLWTSDTHGDEAEQEGGGEN	90%
<i>Drosophila</i>	KACQLAKQAFDDAIAELDTLNEDSYKDSLIMQLLRDNLTLWTSDTQGDEAEQEGGDN	81%
<i>A. thaliana</i>	RACNLAKQAFDEAIAELDTLGEESYKDSLIMQLLRDNLTLWTSDMQDDVAD	65%
<i>C.elegans</i>	DKACQLAKQAFDDAIAELDTLNEDSYKDSLIMQLLRDNLTLWTSDAATDDTDANETEGG	81%
<i>S.cerevisae</i>	KACHLAKQAFDDAIAELDTLSEESYKDSLIMQLLRDNLTLWTSD	70%

Figure 7-20: Alignment of the 14-3-3 zeta/delta proteins found in different species

The sequence of 14-3-3 zeta/delta was aligned for mammals (*Homo sapiens*- human and *Mus musculus*- Mouse), birds (*Gallus gallus*- Chicken), fishes (*Salmo salar*- Salmon), amphibians (*Xenopus laevis*- Frog), insects (*Drosophila melanogaster*- Fly), plants (*Arabidopsis thaliana*), nematodes (*C. elegans*) and yeast (*Saccharomyces cerevisiae*). Differences in the 14-3-3 zeta/ delta or equivalents between the different species are highlighted in blue. PEPITEM or equivalents is shown in red on the sequence and differences between the different species on the PEPITEM sequence are highlighted in yellow. Green highlighted amino acids show differences in amino acids with similar biochemical properties to the amino acids of the human peptide sequence. We found 100% homology for PEPITEM for mice and chicken compared to human; 93% for fish, 92% for the frog, 86% for the fly, 71% for the plant, 92% for *C.elegans* and 78% for yeast.

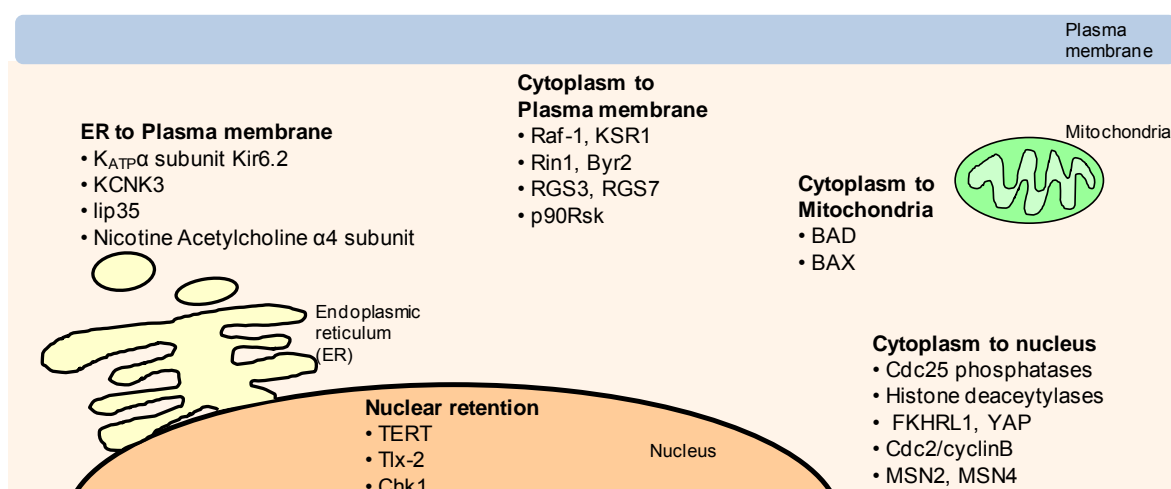


Figure 7-21: 14-3-3 proteins regulate other protein localisation and signalling.

Binding partners whose localisation and functions are regulated by 14-3-3 proteins are listed. (Adapted from Dougherty *et al.*, 2004).

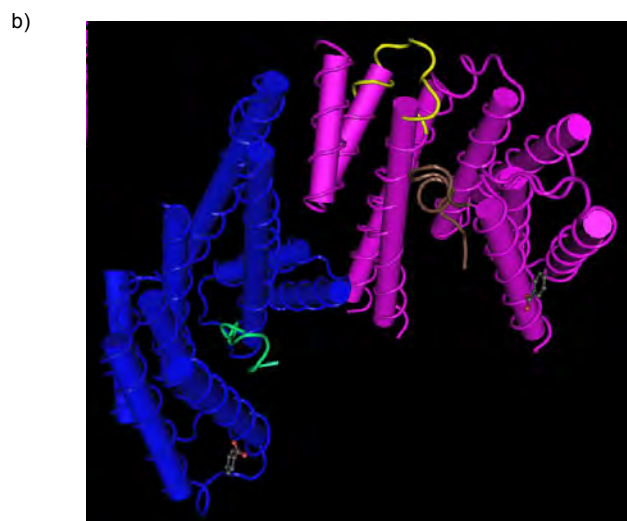
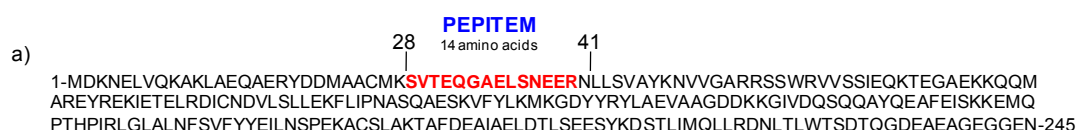


Figure 7-22: Sequence and crystal structure modelling of 14-3-3 zeta/delta protein

(a) 14-3-3 zeta/delta protein is composed of 245 amino acids. PEPITEM is located from amino acid 28 to 41 in the protein sequence. (b) This structure shows a 14-3-3 zeta dimer. Each monomer is composed of nice anti-parallel α -helices. This structure shows the binding of the exoenzyme S peptide in a hydrophobic manner (green and brown residues). Here we aimed to highlight the structural localisation of PEPITEM (yellow residues). The peptide is located in the loop between α -helices 2 and 3 and seems to be relatively exposed. (Structure from Ottmann *et al.*, 2007).

8.CHAPTER 8- GENERAL DISCUSSION

In this thesis, I demonstrate a potential role for the adipokines leptin and adiponectin in the pathophysiology of T1D.

I found higher expression of LEPR on PBMC from T1D but was not able to determine a functional relevance for this difference. However, I was able to determine possible functional relevance for the lower levels of adiponectin receptors on PBMC that has previously been described in our laboratory. I have demonstrated that adiponectin decreases T cell migration across inflamed endothelium, and that the lower adiponectin receptor expression in T1D results in greater transmigration of T cells. I went on to show that adiponectin achieves its effects by stimulating the secretion of a newly identified peptide by B cells. The 14 amino acids peptide, PEPITEM originates from the 14-3-3 zeta/delta protein and induces release of S1P by the endothelium which in turn blocks the trans-endothelial migration of T cells.

These observations not only add to the knowledge of adiponectin involvement in the control of lymphocyte recruitment to the endothelium but also led to the discovery of PEPITEM. PEPITEM holds therapeutic potential for conditions resulting from inappropriate migration of T cells across inflamed endothelium. These would include T1D, rheumatoid arthritis, Crohn's disease, and atherosclerosis. PEPITEM may offer greater therapeutic potential than adiponectin or S1P. Indeed, adiponectin regulates many homeostatic functions, such as lipid and glucose metabolism and insulin sensitivity and is already present at very high levels in the circulation (Arita *et al.*, 1999; Kadowaki, Yamauchi, 2006). S1P regulates the egress of T cells from the SLOs and regulates trafficking of T cells within and into lymph nodes (Cyster, 2005). Use of either adiponectin or S1P as “anti-T cell” recruitment strategies is likely to have significant off-target effects on metabolic and immune function.

Current immunotherapies for T1D aim to deplete populations such as T cells (anti-CD3) and B cells (Rituximab). Despite the success of these therapies to preserve residual beta-cell function, these treatments are associated with undesirable adverse effects and appear to fail over a long-term period (Pescovitz *et al.*, 2009; Chatenoud *et al.*, 1994; Herold *et al.*, 2002; Keymeulen *et al.*, 2005). In contrast to these therapies, we believe that PEPITEM may allow blockade of T cell recruitment in the islets without affecting antibody or T cell responses to pathogens.

Interestingly, Rituximab causes depletion of B cells and this is associated with preservation of beta-cells in T1D, therefore suggesting a negative role of B cells in the disease pathophysiology (Pescovitz *et al.*, 2009). In addition, studies have shown that the B1 B cells subset can specifically promote the infiltration of T cells in the pancreas of diabetic mice (Ryan *et al.*, 2010). In contrast, we show that B cells are crucial to down-regulate T cell recruitment to the inflamed endothelium and might be beneficial to stop beta-cell destruction. These findings are not necessarily contradictory. First, it is unsure which B cells subsets are completely depleted with Rituximab (Browning, 2006). It is therefore essential to identify the subset of B cells capable of secreting PEPITEM. In addition, recent evidence refers to B cells as “not always the bad guys” (Fillatreau *et al.*, 2008). Indeed, studies have identified a subset of B cells able to regulate autoimmune diseases by production of IL-10 and depletion of this subset worsens autoimmune diseases (Fillatreau *et al.*, 2002; Wolf *et al.*, 1996; DiLillo *et al.*, 2010). This subset is referred as regulatory B cells (Breg). Breg have been identified and characterised in mice but only a few studies have looked at Breg in human and the current literature does not provide specific markers to identify Breg in the circulation (DiLillo *et al.*, 2010). Although, we believe that effector B cells (long-life plasma cells and memory B cells) might be responsible for PEPITEM production, this has not been confirmed. In addition, our

panel of markers did not allow identification of Breg on the endothelium as the markers are not yet known.

The discovery of PEPITEM as an endogenous peptide belonging to the 14-3-3 zeta/delta protein is intriguing as this family of proteins is barely involved in the control of lymphocyte transmigration. Indeed, the current literature in this area only demonstrates a role of 14-3-3s to regulate integrin activation in lymphocytes and fibroblasts by binding to their signalling partners (Nurmi *et al.*, 2006). To our knowledge, none of these proteins have been associated with a control of SIP production that would influence T cell migration across the endothelium.

The results outlined in this thesis raise important issues that need further urgent investigation. In particular, we urgently need to assess the pathways linking adiponectin, SIP and PEPITEM, as well as test the therapeutic potential of this in animal studies. A more comprehensive list of experiments this work leads onto is listed following this discussion.

In summary, my work provides new insights in the role of adiponectin in modulating T cells recruitment across the endothelium and we identified an endogenous peptide capable of mediating this effect. These findings suggest an intricate communication between B and T cells, which are already known to interact in the course of immune responses. The ability of B cells to make the peptide may vary in autoimmune diseases and may contribute to the development of disease. Therefore, understanding PEPITEM biology and effect in murine models of autoimmune diseases is imperative. I also show how adiponectin could be involved in the development of T1D, and how it may link the recognised epidemiological association between insulin resistance and the development of this disease.

Further experiments:

Whilst the work outlined in this thesis is informative, it reveals areas that need further investigation. These are listed below:

- **Leptin:**

- ✓ Analyse the effect of leptin on the stimulation of naive versus memory T cells
- ✓ Study the effect of leptin on the behaviour of monocytes on the endothelium as these express high levels of LEPR.

- **Adiponectin and S1P:**

- ✓ Confirm the involvement of the adiponectin receptors in the inhibition of lymphocyte transmigration by adiponectin.
- ✓ Investigate the release of S1P by HUVEC under adiponectin or PEPITEM stimulation: mass spectrometry analysis, SPHKs inhibitors or lentiviral knock-down.
- ✓ Analyse the effect of S1P on T cell migration: integrins localisation and activation and cAMP levels.
- ✓ Measure effect of S1P (under PEPITEM stimulation) on the recruitment of other leukocytes subsets that express S1P receptors. For instance, monocytes express S1P1, 2 and 4. Studies have shown that binding of monocytes to aortas from NOD mice, is increased compared to the wild-type and treatment of the aortas with S1P considerably reduced the adhesion of monocytes (Whetzel *et al.*, 2006). Further studies show that S1P inhibits monocyte adhesion to the endothelium by shifting the apical localisation of integrins to the basal surface (Aoki *et al.*, 2007).

- **PEPITEM**

- ✓ Measure the pharmacology of PEPITEM: acquisition of a radio-labelled peptide and measure its binding affinity to stimulated HUVEC with classical methods of pharmacokinetics.
- ✓ Analyse PEPITEM toxicity *in vivo*.
- ✓ Follow T cell recruitment under PEPITEM treatment in the pancreas: *in vivo* imaging.
- ✓ Identify the endothelial receptor for PEPITEM: biotin pull-down and mass spectrometry.
- ✓ Analyse PEPITEM circulating levels in health and T1D or other autoimmune diseases: mass spectrometry.
- ✓ Synthesis of antibodies raised against PEPITEM
- ✓ Understand how PEPITEM is cleaved from the 14-3-3 zeta/delta protein: proteasome?
- ✓ Test PEPITEM in animal model of autoimmune diseases (Collaboration with Dr Susan Wong): need to determine its pharmacokinetics for appropriate dosing.

9.CHAPTER 9- REFERENCES

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Appendix

Publications

Articles:

- Diabetologia (2012) 55:542-541

What are the health benefits of physical activity in type 1 diabetes mellitus? A literature review.

Chimen M, Kennedy A, Nirantharakumar K, Pang TT, Andrews R, Narendran P.

- Submitted to Diabetes, January 2012

Reduced adiponectin receptor expression by monocytes in human type 1 diabetes associates with pro-inflammatory response to islet antigens.

Pang TTL, Chimen M, Goble E, Benbow GA, Eldershaw SE, Gough SCL, Narendran

Conferences Abstracts:

- IDS May 2009- Poster prize

PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMC) EXPRESSION OF LEPTIN RECEPTORS IS INCREASED IN TYPE 1 DIABETES (T1D)

Myriam Chimen, Terence T Pang, Edward Goble, Suzy Eldershaw, Peter Lane & Parth Narendran

- Diabetes UK March 2010

Differential expression of adiponectin and leptin receptors by immune cells in type 1 diabetes (T1D): a potential link between insulin resistance (IR) and pancreatic islet immunity

Myriam Chimen, Terence T Pang, Edward Goble, Suzy A Eldershaw, Parth Narendran

- **Arpil 2010: Robert's prize for doctoral research poster presentations-** College of medical and dental sciences research and enterprise gala, University of Birmingham.

- American Diabetes Association June 2010

Differential expression of adiponectin and leptin receptors by immune cells in Type 1 Diabetes (T1D): a potential link between insulin resistance (IR) and pancreatic islet autoimmunity

Myriam Chimen, Terence T Pang, Edward Goble, Suzy A Eldershaw, Parth Narendran

- Diabetes UK March 2011

LYMPHOCYTE RECRUITMENT ACROSS INFLAMED ENDOTHELIUM IS RELEASED FROM THE INHIBITORY EFFECTS OF ADIPONECTIN IN TYPE 1 DIABETES (T1D)

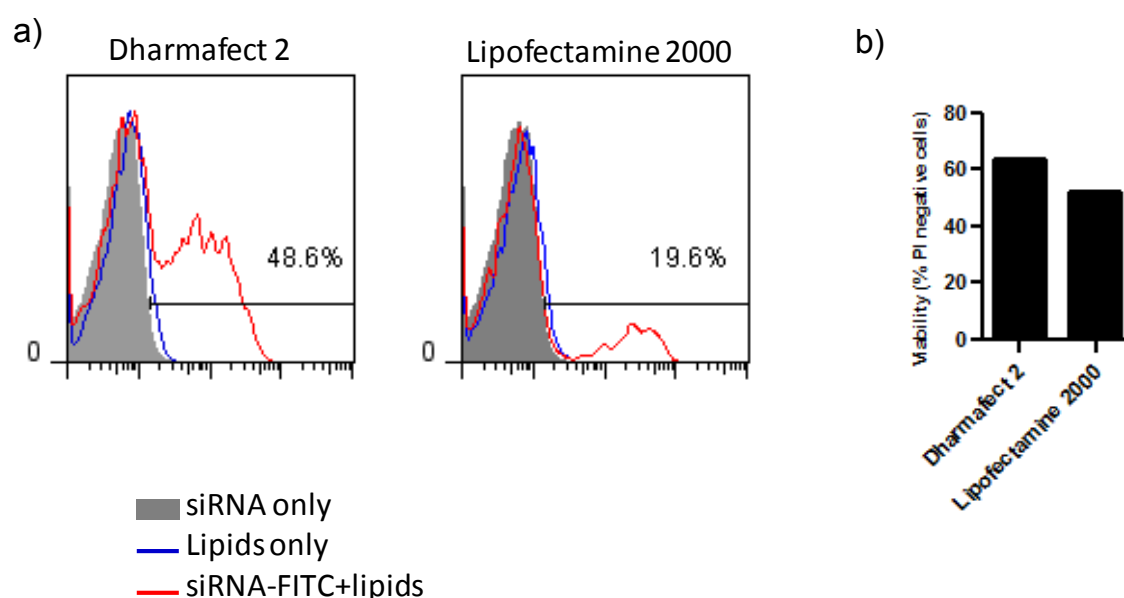
Myriam Chimen, Helen M. McGettrick, G. Ed Rainger & Parth Narendran

- **April 2011: First prize for doctoral research poster presentation-** College of medical and dental sciences research and enterprise gala, University of Birmingham.

Appendix

Sabbatical at the Hagedorn Institute (Novo Nordisk) in Copenhagen

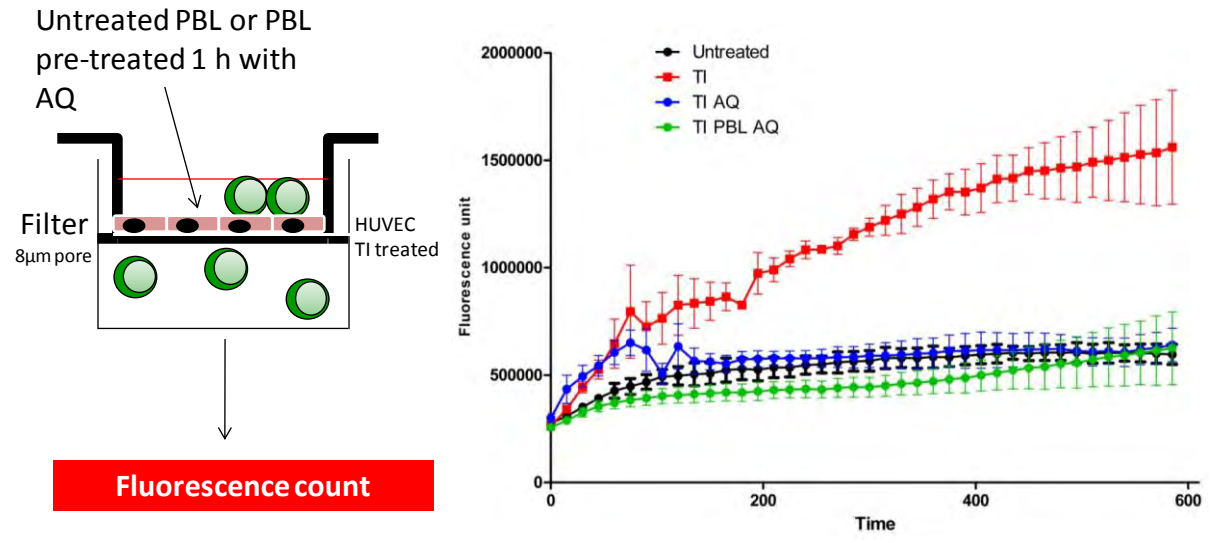
From October to December 2010, I joined the Hagedorn Institute under Anette Sams supervision in the context of our collaboration with Novo Nordisk. During this sabbatical, I started the optimisation of a method for transfecting lymphocytes with siRNA (**Appendix Figure 1**). I also reproduced some of my *in vitro* data showing that adiponectin inhibits the trans-endothelial migration of lymphocytes, using a transwell assay with fluorescently labelled lymphocytes (**Appendix Figure 2**).



Appendix Figure 1: Development of a method to transfect lymphocytes with siRNA

(a) PBL were transfected with siRNA-FITC using Dharmafect 2 or Lipofectamine 2000, two lipid-mediated delivery methods. Transfection efficiency was measured by flow cytometry. (b) Viability was measured using a PI exclusion method.

Appendix



Appendix Figure 2: Effect of adiponectin on PBL transmigration in a transwell system

(a) Schematic representation of the transwell system. HUVEC were cultured in low-serum conditions and stimulated with $\text{TNF-}\alpha/\text{IFN-}\gamma/\text{adiponectin}$ (TI or TI AQ). PBL were isolated and in some conditions pre-treated with adiponectin for 1 hour and added on the endothelium (TI PBL AQ). The transwell plate is placed in a fluorescent reader kept at 37°C and fluorescence is measured only in the bottom chamber (only possible because the filter and top chamber are made of black plastic). (b) In both HUVEC and PBL treatment, adiponectin significantly reduced PBL transmigration in the bottom chamber over time.